Molecular cloning and characterisation of G-protein-coupled receptors from the pituitary gland

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Contents

Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree. All experiments were performed at the Medical Research Council Reproductive Biology Unit in Edinburgh.

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Abstract

This thesis describes molecular cloning and characterisation studies of two G-protein coupled receptors (GPRs), the thyrotrophin-releasing hormone receptor (TRH-R) and the dopamine D2 receptor (D2-R). These two receptors are both expressed in the pituitary gland where they mediate the actions of TRH and dopamine on the regulation of prolactin and thyrotrophin (TSH) secretion. A human TRH-R clone was isolated from a pituitary cDNA library (Duthie et al., 1993a). Sequence analysis identified the clone as a TRH-R encoding a putative protein of 398 amino acids. The human pituitary TRH-R had high sequence homology with the rat and mouse TRH-Rs, except at the 3' end of the carboxy (COOH) tail. In this region, residue 392 is a common point of sequence divergence, after which the human, rat and mouse TRH-Rs encode variant amino acids. The COOH tail of GPRs has been implicated in functionally significant events including receptor phosphorylation, desensitisation and possibly second messenger coupling. The human TRH-R clone was transiently expressed in COS-1 cells and showed high affinity receptor binding using [^{3}H](3-Met-His^{2})TRH. Stimulation of these cells with TRH produced a typical phosphoinositide response with mobilisation of intracellular calcium. In addition to its role as a pituitary releasing factor, TRH appears to act as a neurotransmitter/neuromodulator in brain, retina and the gastro-intestinal and reproductive systems. The distribution of TRH-R messenger RNA (mRNA) was therefore studied by *in situ* hybridisation and Northern blot analysis, in several human tissues, using radio-labelled probes based on the human TRH-R cDNA. Positive hybridisation to the TRH-R probe was found in glandular epithelium and stroma of human prostate tissue (benign and malignant) using *in situ* hybridisation. The expression of TRH-R mRNA transcripts in the prostate was further supported by Northern blotting, suggesting a possible biological role for TRH in human prostate. The identification of TRH-Rs with variant COOH tails raised the possibility of alternative splicing in the TRH-R gene in this region of the receptor. To answer this question, a TRH-R gene was cloned from a mouse genomic library (Duthie et al., 1993b). Characterisation of the gene focused on the COOH terminal region. A consensus splice junction was identified at the common point of sequence

divergence (amino acid 392) found in the mouse, rat and human TRH-R COOH tails. This splice site separated a 228bp exon (putative Exon 3), encoding the final amino acid and stop codon of the mouse TRH-R, from the rest of the coding region. This exon corresponded exactly to a deletion in the rat TRH-R cDNA, which was 19 amino acids longer than the mouse cDNA, in spite of the deletion. The nucleotides encoding these extra 19 residues were found on Exon 4 of the mouse gene, suggesting a possible splicing event in this region. To date there is no evidence of a second form of the mouse receptor. The mouse D2-R gene was also cloned and characterised. The gene was structurally similar to the rat D2-R gene with the coding region contained on 7 exons, spanning approximately 12kb of the genome. The first exon, encoding 5' untranslated sequence, was not identified due to the presence of an unusually large intron between the first and second exons of the receptor. Like the rat D2-R, but unlike the human receptor, the mouse gene encoded a variant donor splice site (gc instead of gt) on Exon 4. Ultimately, the mouse TRH-R and D2-R genomic clones may be used to study the significance of possible splicing events in the regulation of prolactin secretion using transgenic mouse models. Information relating to the sequence and structural organisation of the genes encoding these GPRs will facilitate the identification of mutant receptors which may be involved in disease states. Analysis of receptor structure/function relationships will facilitate the study of ligand/receptor interactions, leading to the design of agonists and antagonists selective for a particular receptor subtype.

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Duthie, S.M., Taylor, P.L. & Eidne, K.A. (1993b) Journal of Molecular Endocrinology 11:141-149.

(Approximately 40,000 words in main text)

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Abbreviations

Ala	alanine
Amp	ampicillin
Arg	arginine
Asn	asparagine
Asp	asparatic acid
β-gal	β-galactosidase
βAR	β-adrenergic receptor
BSA	bovine serum albumin
cDNA	complementary DNA
CL	cytoplasmic loop
COOH	carboxy
Cys	cysteine
D2-R	dopamine D2 receptor
DA	dopamine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EL	extracellular loop
EtBr	ethidium bromide
FCS	foetal calf serum
FISH	fluorescence *in situ* hybridisation
FSH-R	follicle stimulating hormone receptor
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GnRH-R	gonadotrophin-releasing hormone receptor
GPR	G-protein-coupled receptor
His	histidine
Ile	isoleucine
kb	kilobase
Leu	leucine
LH-R	luteinising hormone receptor
LMP	low melting point gel

Lys	lysine
M	molar
mM	millimolar
Met	methionine
ml	millilitre
µl	microlitre
mRNA	messenger RNA
NH_2	amino
Oligo	oligonucleotide
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
Phe	phenylalanine
Pro	proline
RE	restriction enzyme
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Ser	serine
SSC	sodium salt citrate
TBE	tris, borate, EDTA
TEMED	N,N,N',N'-Tetra-methylethylenediamine
Tet	tetracycline
Thr	threonine
TM	transmembrane
TRH	thyrotrophin-releasing hormone
TRH-R	TRH receptor
tRNA	transfer RNA
Trp	tryptophan
TSH	thyrotrophin (thyroid stimulating hormone)
Tyr	tyrosine
u/v	ultra-violet
Val	valine
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1 Introduction

One of the fundamental requirements of living organisms is the need to communicate with the external environment and to transmit the information received to internal signalling systems. The ability to communicate requires the same three basic steps in all organisms, from simple yeasts to complex, multicellular mammals with sophisticated nervous systems. Firstly, an external stimulus is received by a specific receptor molecule, secondly, the information must be transmitted to the interior of the cell and finally the message has to be processed such that the cell produces an appropriate physiological response.

A wide variety of external stimuli are known to exert their biological effects via membrane-bound receptors coupled to intracellular guanine-nucleotide binding proteins, or G-proteins. These G-protein-coupled receptors (GPRs) have a common seven membrane-spanning domain secondary structure and belong to an increasingly large superfamily of molecules that respond to such diverse stimuli as light, odourants, hormones, neurotransmitters and growth factors. Receptors that receive the yeast MATa or MATα mating pheromones are members of the GPR superfamily (Blumer et al., 1988) while in mammals, GPRs are known to be involved in almost every process required for the normal functioning of higher organisms, including the transmission of information to the visual and olfactory systems, to endocrine pathways and to the nervous and cardiovascular systems.

Many drugs used in clinical practice bind and activate GPRs, making the study of these receptor structures, and their mechanisms of interaction with external stimuli and internal signalling systems, of prime importance. Initially, receptors were classified using pharmacological tools which characterised receptors with respect to ligands that acted as agonists or antagonists. These methods were able to discriminate between receptors that produced different responses when activated by the same ligand, for example the alpha- and beta-adrenergic receptors (Alquist, 1948), and the dopamine D1 and D2 receptors (Kebabian & Calne, 1979), but were unable to resolve the confusion caused by the ambiguous pharmacological profiles and overlapping distribution patterns of some receptors.

The explosion of recombinant DNA technology in the 1980s has enabled receptors to be cloned and characterised using transfected cell lines, without first identifying the ligand. Sequence analysis has confirmed the existence of multiple receptor subtypes for several receptors, including the muscarinic, adrenergic and dopamine receptors, an important consideration when designing drugs specific for a particular pathway. There have been several recent reports of GPR mutations that are directly responsible for conditions such as familial male precocious puberty (Shenker et al., 1993) and hyperfunctioning thyroid adenoma (Parma et al., 1993) and thus sequence information is also of first importance when assessing the involvement of GPRs in disease.

GPRs play a major role in reproductive biology and are involved in the regulation of lactation and fertility. Hypothalamic hormones controlling the release of prolactin, follicle stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary gland all act via GPRs. These include the dopamine D2-receptor (Bunzow et al., 1988), the thyrotrophin-releasing hormone (TRH) receptor (Straub et al., 1990), and the gonadotrophin-releasing hormone receptor (Tsutsumi et al., 1992) amongst others. The LH receptor (McFarland et al., 1989) and FSH receptor (Sprengel et al., 1990) which mediate the secretion of oestrogens and androgens in the ovary and testis respectively, are also seven membrane-spanning domain GPRs.

Researchers are becoming increasingly aware that results obtained using cloned receptors in transfected cell lines may not accurately represent the *in vivo* situation (Yamada et al., 1992), while marked species differences in the responses of GPRs to different synthetic analogues have been demonstrated (Oksenberg et al., 1992). These observations stress the importance of not only studying human GPRs, but also of characterising receptors in appropriate animal models.

One aim of this research project was to clone and characterise the human TRH receptor (TRH-R) cDNA (Chpt. 4) in order to compare its structure/function relationships with the previously cloned mouse (Straub et al., 1990) and rat TRH-R (Zhao et al., 1992; de la Peña et al., 1992a,b; Sellar et al., 1993). The chromosomal localisation of the human TRH-R was also undertaken in collaboration with Professor Mike Connor's group at the Duncan Guthrie Institute in Glasgow (Chpt. 4).

Until recently, studying receptor distribution has been difficult due to the lack of available antibodies, and low levels of receptor expression. The cloned human receptor was therefore developed for use as a sensitive probe to localise TRH-R mRNA to various human tissues by *in situ* hybridisation and Northern blot analysis (Chpt 5).

The analysis of receptors *in vivo* as well as *in vitro* will provide more accurate information on mechanisms of receptor function and control and to this end the cloning and characterisation of the mouse TRH-R (Chpt. 6) and dopamine D2-R (Chpt. 7) genomic structures was undertaken. Knowledge of genomic splicing mechanisms and of regulatory regions such as promotors and enhancers will help to understand receptor regulation and expression. Ultimately, modified receptor genes may be expressed in transgenic animal models to achieve a more realistic picture of GPR function than can be obtained by using transfected cell lines in isolation.

Chapter 2 attempts a general review of the relevant literature providing a background for the work undertaken. Each experimental chapter includes an introduction encompassing recent literature on the particular topic, together with specific methods used. General techniques that are relevant to more than one section are described in Chpt. 3. The final chapter discusses the aims and achievements of the project in the context of current research and suggests possible areas of future research.

2 Literature Review

2.1 Neuropeptides and neurotransmitters

In the 1930s, work by Ernst Scharrer and colleagues (Reichlin, 1987) suggested the concept of neurosecretion. They realised that certain neurons in the brain/neurohypophyseal system resembled cells of endocrine glands in that they could secrete chemical transmitters directly into the circulation. It was known that the rapid transmission of electrical impulses along neuronal axons in the nervous system required the release of chemical neurotransmitter from the axon terminal across a synapse, or close point of contact, between two neurons. Endocrine tissues, on the other hand, were known to secrete their hormones directly into the blood stream which transported them to target organs throughout the body. The discovery that neurons could secrete chemical transmitters into the vascular system seemed to combine these two processes and the chemical transmitters of the hypothalamus were therefore termed neurohormones, and the new science, neuroendocrinology.

2.2 The Hypothalamic-Pituitary Axis

2.2.1 The pituitary gland

Pioneer work by Green and Harris in the 1940s introduced the concept of the hypothalamic-pituitary axis (Fig. 2.1). They demonstrated the direction of blood flow from the hypothalamus to the pars distalis of the adenohypophysis in living rats (Green & Harris, 1949) which supported the concept of neurovascular control of the anterior pituitary (Green & Harris, 1947). It was then proposed that neurons located in the hypothalamus might secrete certain humoral substances into the portal circulation to regulate normal function of the pituitary gland (Harris, 1955). The nature of the substance(s) was unknown, it was even suggested that the hypothalamus supplied only a general stimulation to the anterior pituitary, with specific control being provided by feedback from the target organs of the pituitary hormones (Harris, 1955).

The knowledge that the posterior 'lobe' of the pituitary was not a gland, but was composed of nerve cell endings with cell bodies originating

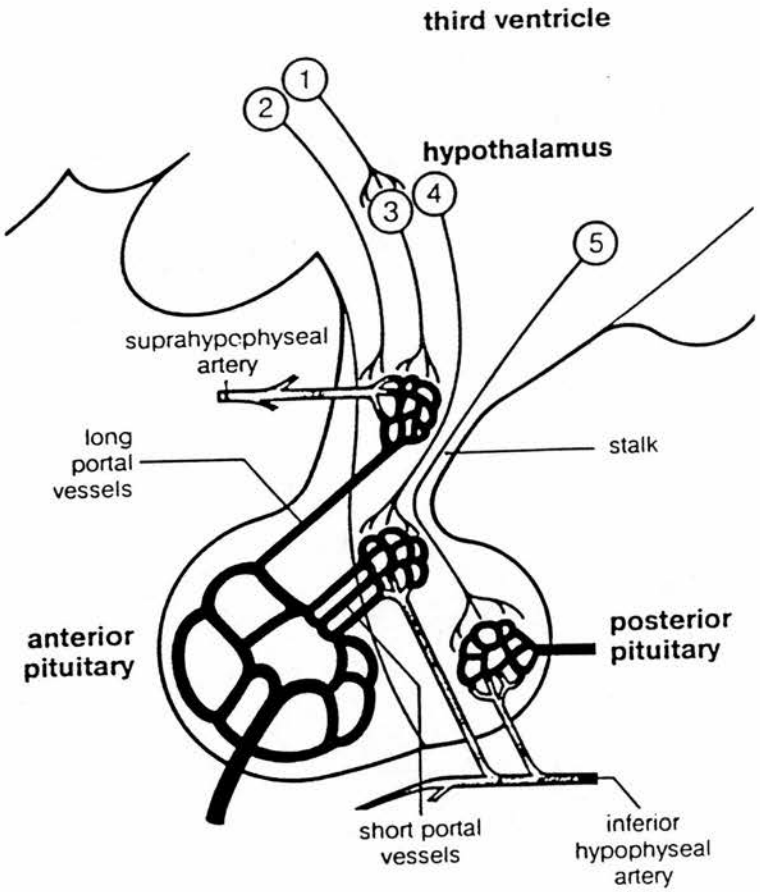


Fig. 2.1

Diagrammatic representation of the hypothalamic-pituitary axis. The releasing or inhibiting factors of the hypothalamus are synthesised within the nuclei of the hypothalamus (1-4) and transported to the median eminence and then to the anterior pituitary via the dense capillary network and long portal veins. The hypothalamic hormones bind to specific receptors on the cells of the anterior pituitary leading to the release or inhibition of the pituitary trophic hormone secretion. The products of the posterior pituitary are synthesised in the supra optic and paraventricular nuclei (5). After packaging, they are transported by axoplasmic flow to the nerve terminals in the posterior pituitary where they are released directly into the circulation to act as classical neurohormones on distinct target sites (adapted from Reichlin, 1987).

in the hypothalamus, which released their hormones directly into the circulation, supported the concept of a hypothalamic-pituitary axis. The neurohypophyseal hormones, oxytocin and vasopressin, were purified in 1950 and 1954 respectively, from the posterior lobe, by du Vigneaud and co-workers, representing the first direct evidence of an endocrine function of the hypothalamus (Brownstein et al., 1980).

2.2.2 Hormones of the anterior pituitary

Anterior pituitary reproductive hormones controlled by the hypothalamus include luteinising hormone (LH) and follicle stimulating hormone (FSH) secreted by gonadotrophs and prolactin (PRL) secreted by lactotrophs (Fig. 2.2). LH and FSH are released together and act synergistically, promoting ovulation and the secretion of oestrogens from the ovary in females, and sperm production and androgen secretion in males. PRL is a lactogenic hormone which stimulates and maintains lactation in mammals, and (in some) the secretion of progesterone from the corpus luteum.

Metabolic hormones of the anterior pituitary include thryotrophin stimulating hormone (TSH) secreted by thyrotrophs, adrenocorticotrophic hormone (ACTH) secreted by corticotrophs and growth hormone (GH) secreted by somatotrophs (Fig. 2.2). TSH stimulates the growth of the thyroid gland, the uptake of iodine and the secretion of its hormones. Stress results in the release of ACTH which then stimulates the synthesis and release of corticosteroid hormones from the adrenal cortex, melanin production in pigment cells and lipolysis in fatty tissue. Finally, GH stimulates skeletal and general body growth and also potentiates the effects of other pituitary hormones.

2.2.3 The Hypothalamus

The hypothalamus is formed from the thickened floor and sides of the posterior part of the vertebrate forebrain, and is situated below the third ventricle of the brain and above the pituitary gland (Fig. 2.1). The maintenance of a stable internal environment requires the co-ordinated regulation of numerous biochemical signals. These signals converge upon the neuronal systems of the hypothalamus, which respond by releasing

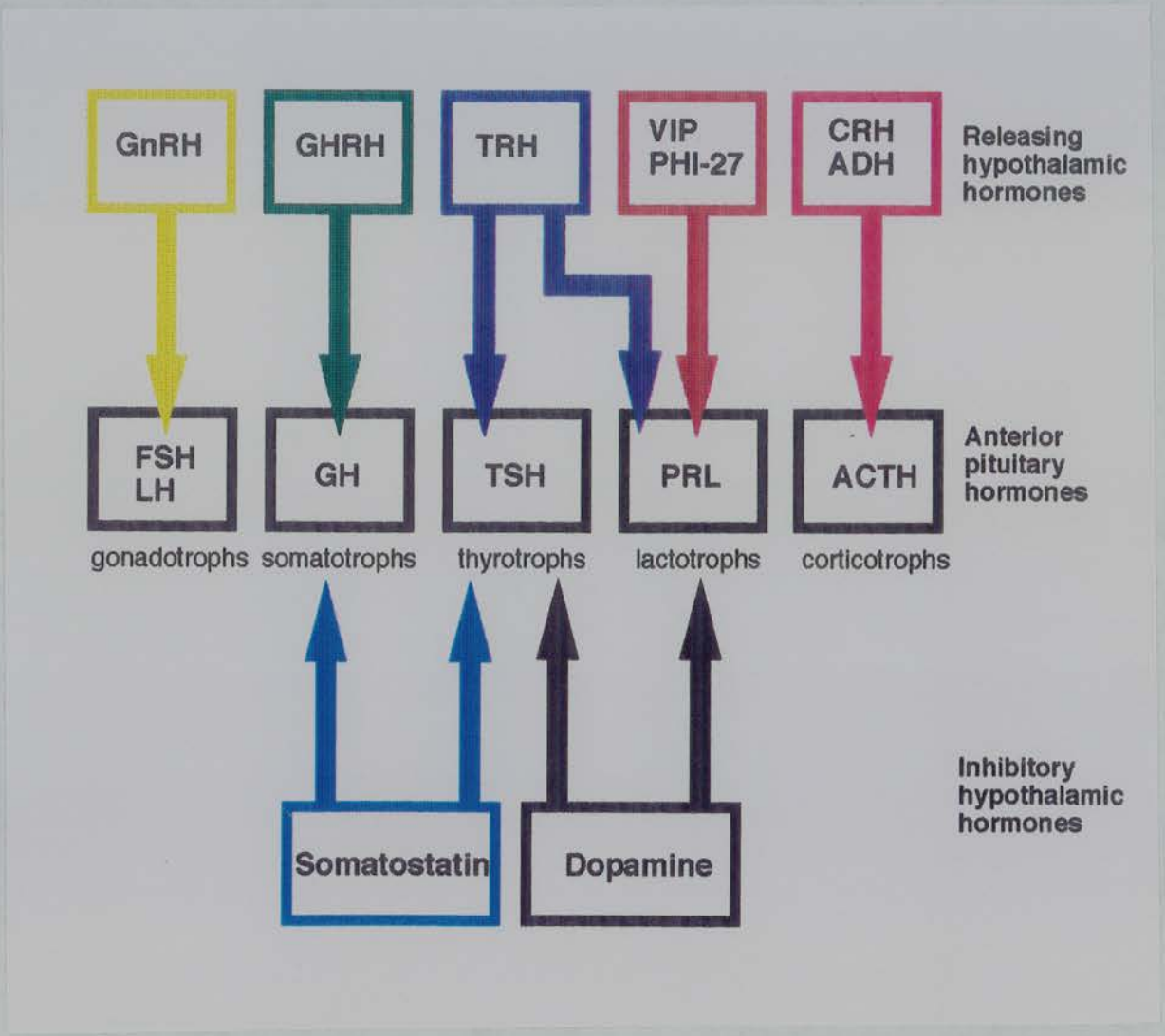


Fig. 2.2

The hormones produced in the anterior pituitary and the hypothalamic hormones that regulate their secretion (Adapted from Reichlin, 1987).

neurohormones into the pituitary portal circulation, resulting in hormonal secretions by the pituitary gland. This in turn, elicits the appropriate metabolic responses. The hypothalamus, therefore acts as an intermediary between the neuronal circuitry of the brain and the endocrine secretion of hormones from the pituitary gland into the general circulation (Reichlin, 1987; Yen, 1991).

2.2.4 Hypopthalmic Hormones

The first of these hormones to be described was corticotrophin releasing factor (CRF, now called CRH) in 1955 (Saffran et al., 1955) which stimulated the release of ACTH from pituitary fragments maintained in organ culture. Thyrotrophin releasing factor (TRF, now called TRH), was the first neurohormone to be isolated and purified (Guillemin, 1971). This discovery paved the way for the purification of the other major hypophysiotrophic hormones which included gonadotrophin-releasing hormone (GnRH), (Matsuo et al, 1971), somatostatin (Brazeau et al., 1973), CRH, (Vale et al., 1981), and growth hormone releasing hormone (GHRH), (Guillemin et al., 1983).

These peptide hormones were thought to be unique to the hypothalamic-pituitary axis, but many have now been found in various regions of the brain and spinal cord. They have also been discovered in the autonomic nervous system, in the gastro-intestinal, reproductive and respiratory tracts and in the placenta (Yen, 1991a). Such peptides may have been involved in cellular communication before the development of neuronal or endocrine systems (Krieger, 1983).

The catecholamines (biogenic amines) - dopamine (DA), noradrenaline (norepinephrine) and adrenaline (epinephrine), classified as neurotransmitters, may also be considered as hypothalamic hormones because they are present in hypophyseal portal blood, and affect pituitary hormone secretion (Paradisi et al., 1989).

2.2.5 Hormonal regulation of the anterior pituitary gland

Each of the known anterior pituitary hormones is regulated by hypothalamic factors interacting with specific receptors on their target cells (Fig. 2.2). One hypothalamic hormone may regulate more than one pituitary hormone, for example, TRH stimulates the release of TSH and

prolactin and in some disease states, such as acromegaly and Cushing's disease, can produce paradoxical rises in GH and ACTH, respectively (Hershman, 1974; Jackson, 1982). Also, one pituitary hormone may be regulated by more than one hypothalamic hormone, as hypothalamic releasing and inhibiting factors interact with each other to exert dual control. For example, the main control over prolactin secretion exerted by the hypothalamus is a tonic inhibition by DA, but prolactin release is stimulated by TRH, vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) (Fig. 2.2).

Neurons secreting hypothalamic hormones are themselves influenced by neuropeptides, neurotransmitters such as DA, and feedback effects of various pituitary hormones (Krieger & Liotta, 1979; Page, 1983). The anterior pituitary hormones are also regulated by feedback effects, generally from circulating levels of hormones from their target glands.

The aim of this research project has been to study anterior pituitary gland, G-protein-linked receptors for neurotransmitters and neuropeptides, and has chosen to focus on the thyrotrophin-releasing hormone, TRH (a neuropeptide) receptor and the dopamine (a neurotransmitter) D2 receptor. These hormones are involved in the inhibition (dopamine) and stimulation (TRH) of prolactin and TSH secretion, and a brief outline of their origins and functions is given below.

2.3 Thyrotrophin-releasing hormone

A hypothalamic releasing factor for thyrotrophin, (thyroid stimulating hormone, TSH) was proposed in the 1950s (Greer, 1951) and was the first of these peptides to be purified, chemically characterised, synthesised and administered to humans to alleviate disruptions of the thyroid axis (Guillemin, 1971; Schally et al., 1966). This peptide was named thyrotrophin-releasing factor (TRF) and is now more commonly known as thyrotrophin-releasing hormone (TRH) since it is released directly into the pituitary portal circulation from the hypothalamus, to stimulate the release of TSH and also prolactin from its target organ, the anterior pituitary gland (Steiner et al., 1970; Tashjian et al., 1971). The characterisation of porcine TRH (Folkers et al., 1969) and ovine TRH (Burgus et al., 1970) showed that TRH was a weakly basic tripeptide

amide with the structure pyroglutamyl-histidyl-proline-amide (pyroGlu-His-Pro-NH_2) (Fig. 2.3), and that the cyclised glutamic acid residue and an intact amide were essential for activity.

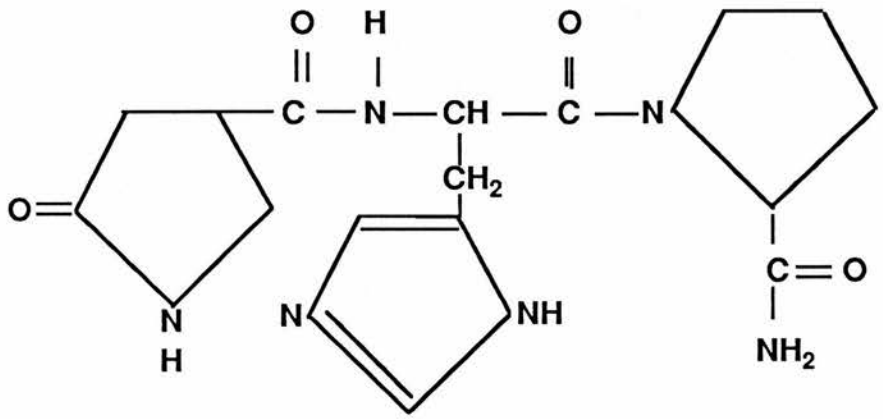


Fig. 2.3
The chemical structure of TRH [pyroGlu-His-Pro-NH_2] (from Folkers et al., 1969).

2.3.1 Structure, synthesis and metabolism of TRH

In common with other neuropeptides such as the enkephalins and opioids (Krieger, 1983), TRH was shown to be derived from the post-translational cleavage of a larger precursor molecule, or pro-hormone (Richter et al., 1984; Jackson & Wu, 1985; Nillni et al., 1993). Several TRH-like peptides have recently been characterised, some of which may be produced by alternative post-translational processing of the TRH pro-hormone. Others appear to be the products of distinct genes (Bilek et al., 1992; Fuse et al., 1990).

TRH has a short half-life and is rapidly degraded in tissues and serum into TRH-free acid (diamido-TRH), histidyl-proline-diketopiperazine (His-Pro-DKP) and constituent amino acids (Bower et al., 1978; Jackson et al., 1979; Jikihara et al., 1993). His-Pro-DKP is an active metabolite of TRH and may, or may not, have a possible role as a dopamine-uptake blocker in the rat hypothalamus (Peters et al., 1985; Jikihara et al., 1993).

2.3.2 Localisation of TRH to the hypothalamus

TRH has been localised to the hypothalamus and stalk medium eminence with particularly high concentrations in the paraventricular nucleus, the classic 'thyrotrophic area' of the hypothalamus (Jackson & Reichlin, 1974; Lechan & Jackson, 1982). Ablation of the thyrotrophic area induces hypothyroidism in the rat as a result of 70% depletion of total hypothalamic TRH (Jackson & Reichlin, 1977). TRH is also present in the nerve fibres of the posterior pituitary, where its concentration is second only to the hypothalamus (Jackson & Reichlin, 1974; Lechan & Jackson, 1982). Ablation of the thyrotrophic area also reduces posterior pituitary TRH, thus the hypothalamus is probably the source for both anterior and posterior pituitary TRH (Jackson & Reichlin, 1977). It has been suggested that TRH may be also be produced by the anterior pituitary gland itself, and may act as a paracrine regulator of anterior pituitary hormonal secretion as well as having an endocrine function (Childs et al., 1976).

2.3.3 TRH and the hypothalamic-pituitary-thyroid axis

In the 1940s, Hoskins (1949) proposed a 'servo' or feedback mechanism for the regulation of pituitary TSH release by thyroid hormones. It was known that increased levels of 3,5,3'-triiodothyronine (T_3), following increased secretion of TSH, resulted in the selective inhibition of TSH from the anterior pituitary, and decreased T_3 release. Thus steady state levels of circulating thyroid hormone were maintained (Fig. 2.4).

Additional control over pituitary-thyroid function was proposed by Green & Harris (Harris, 1955) when a role for the hypothalamus in regulating pituitary hormone release was discovered. Greer produced direct evidence to support this theory in 1951 (Greer, 1951), when he demonstrated that inducing lesions in the hypothalami of rats prevented the stimulation of pituitary TSH release. This work supported the existence of a hypothalamic TSH-releasing factor. This factor (TRH) (Guillemin et al., 1971), was shown to act as a sort of 'thermostatic secondary regulator' of TSH, by counter-balancing the negative feed-back effects of the thyroid hormones, the primary regulators of TSH secretion, on the anterior pituitary (Bowers et al., 1967). TRH is a potent hypophysiotrophic factor, which binds to specific receptors on pituitary thyrotrophs, activating the phospholipase C-inositol phosphate

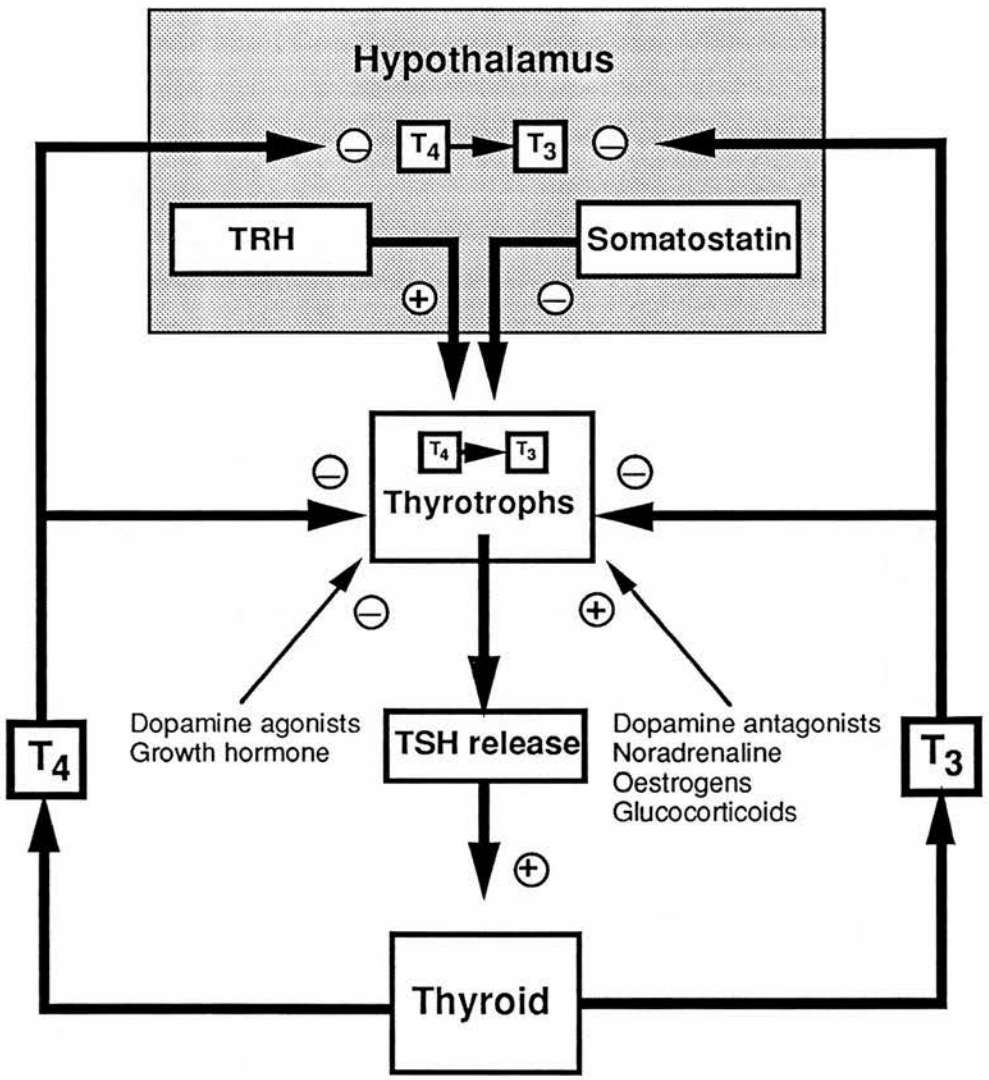


Fig. 2.4

Diagrammatic representation of the neuroendocrine regulation of TSH secretion. The rate of secretion of TSH is modulated by a positive signal from the hypothalamus (TRH) and two negative feedback mechanisms, one from brain (somatostatin) and one from thyroid (thyroid hormones T_3/T_4). The thyroid hormones act on pituitary and hypothalamus. The thyroid hormones have a negative effect on the hypothalamus with respect to the control of TSH secretion (Adapted from Reichlin, 1987).

intracellular second messenger signalling system (see section 2.6.8) resulting in a characteristic TSH surge.

2.3.4 Feedback regulation of hypothalamic TRH

As well as their negative feed-back effects on the pituitary gland, the thyroid hormones are involved in feedback at the hypothalamic level. Administration of T_3 to the hypothalamus of monkeys inhibited TSH secretion from the pituitary (Belchetz et al., 1978), by apparently inhibiting the release of TRH and/or regulating its breakdown (Jackson et al., 1979). However, the thyroid hormones may be acting instead to stimulate the release of neurotransmitters, including dopamine and somatostatin, known to be involved at both TRH and TSH levels of control (Montoya et al., 1979; Berelowitz et al., 1980; Chen & Ramirez, 1981; Smythe et al., 1982).

TSH release is inhibited by dopamine acting directly on thyrotrophs (Besses et al., 1975) and by somatostatin, the characteristic inhibitor of GH (Maeda & Frohman, 1980), and is stimulated by noradrenaline (Montoya et al., 1979). Extra-hypothalamic factors involved are the glucocorticoids, which inhibit TSH release (Re et al., 1976) and oestrogens which stimulate it, and have been shown to increase the number of TRH-Rs on thyrotrophs (de Léan et al., 1977). TSH itself, may also be involved in a 'short-loop' feedback, acting on the hypothalamus to influence TRH release (Roti et al., 1978).

2.3.5 TRH and regulation of prolactin release

The synthesis and release of prolactin is under complex dual regulation by the hypothlamus, involving the interaction of prolactin-inhibiting factors (PIFs) such as dopamine and prolactin-releasing factors (PRFs) including TRH. The mechanisms of this regulation are discussed in section 2.5.

2.3.6 TRH and endocrine disease

The characteristic response of TSH to intravenous injection of TRH (Fig. 2.5) has resulted in the use of TRH as an indicator of several thyroid-related disorders (Snyder et al., 1974; Hershman, 1974). The TRH test is based on the direct action of T_3/T_4 on the pituitary gland. If the thyroid hormones are present in excess, TRH generally has no effect on TSH

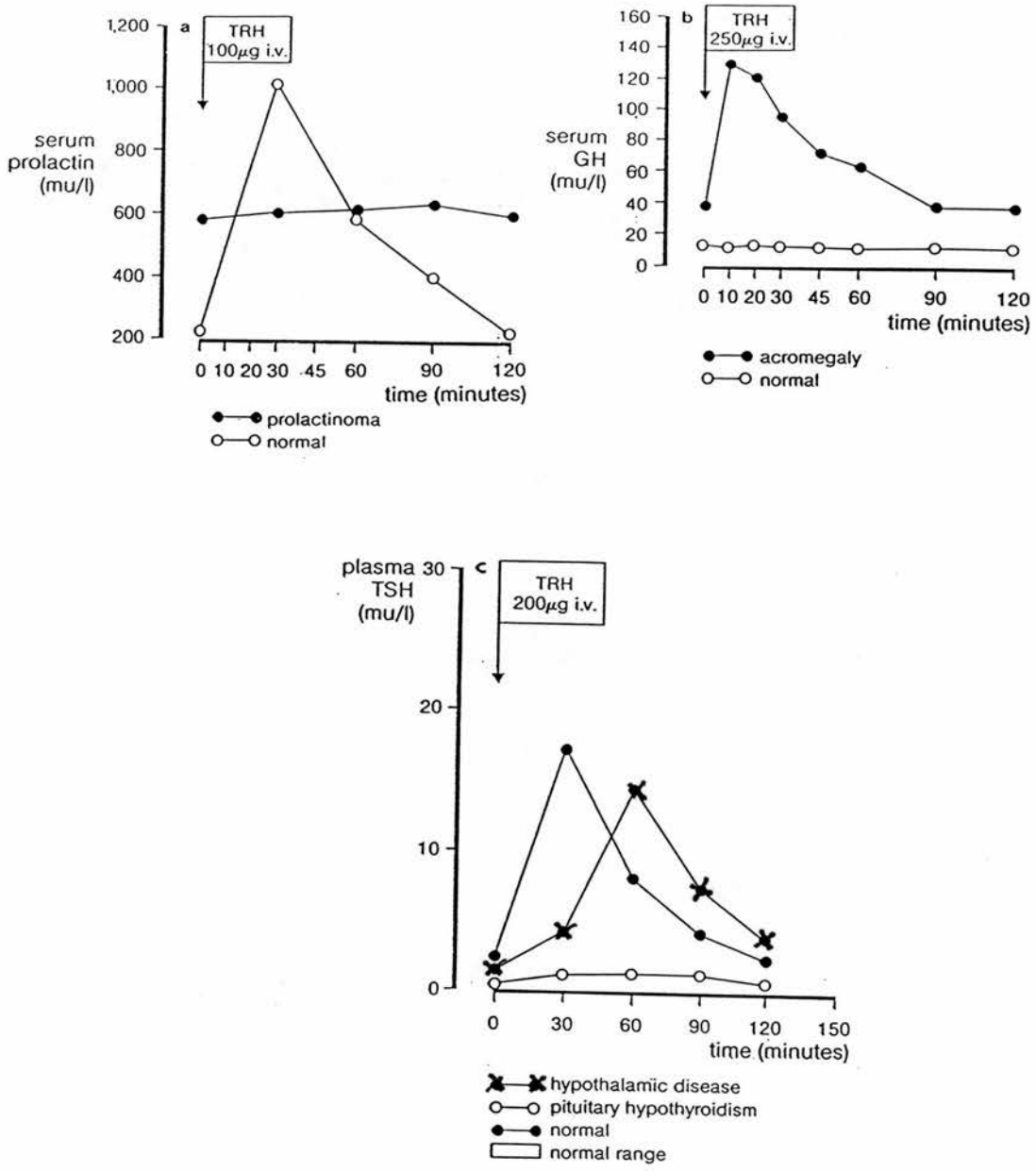


Fig. 2.5

Graphs showing the normal and disease responses to the TRH test. Graph (a) shows the failure of the pituitary to respond to TRH in prolactinoma. Graph (b) shows the paradoxical release of GH in response to TRH, which occurs in acromegaly. Graph (c) shows the delayed TSH response to TRH in hypothalamic disease.

release, since TSH would be maximally inhibited by T_3/T_4 (Reichlin, 1987). The greatest value of TRH in clinical medicine has been in the differential diagnosis of thyrotoxicosis (primary hyperthyroidism) in borderline cases of mild thyroid overactivity, which show no TSH response to the test (Hershman, 1974; Burrow, 1991).

It was hoped that the test would be able to differentiate between hypothyroidism due to a defect at the hypothalamic level (affecting TRH), or at the pituitary level (blockade of response to TRH, perhaps due to defective TRH-Rs) or at the thyroid level (affecting release of the thyroid hormones). However, the TRH test has had limited use in this area due to the wide variety of factors involved in regulating the hypothalamic-pituitary-thyroid axis, which can affect the response of TSH (Snyder et al., 1974; Cobb et al., 1981).

TRH stimulates prolactin release (see section 2.5), but in patients with prolactin-secreting pituitary adenomas (prolactinomas), a blunted prolactin response to the TRH test generally occurs, see Fig. 2.5 (Kleinberg et al., 1977). Specific receptors for TRH were demonstrated on the membranes of prolactinoma cells, suggesting that the blunted response to TRH is probably due to the maximal stimulatation of prolactin, rather than a lack of functioning TRH-Rs (le Dafniet et al., 1983). It was hoped that the TRH test would be able to separate prolactinomas from other causes of hyperprolactinaemia such as primary hypothyroidism (low levels of T_3/T_4 reduce the inhibitory effect on prolactin secretion). However, a blunted prolactin response can also be found in other diseases and the test was found to lack specificity (Kleinberg et al., 1977).

Abnormal responses to TRH have also been observed in other forms of endocrine disease (Fig. 2.5). In normal circumstances, TRH has no effect on GH release from somatotrophs in the anterior pituitary, however, a paradoxical rise in GH in response to TRH has been observed in some patients suffering from acromegaly, diabetes mellitus or primary hypothyroidism in children (Maeda et al., 1975; Jackson, 1982; Scanlon & Hall, 1989). TRH can also stimulate the release of ACTH from pituitary corticotrophs in some patients with Cushing's disease and Nelson's syndrome (Krieger & Condon, 1978).

2.3.7 Extra-hypothalamic TRH

Once specific antibodies had been developed for detecting TRH by immunoassay and immunohistochemistry, the peptide was found, rather surprisingly, to exist in regions outside the classic thyrotrophic area of the hypothalamus. TRH was identified in virtually all areas of the brain (Jackson & Reichlin, 1974; Winokur & Utiger, 1977; Brownstein et al., 1977) and spinal cord (Hokfelt et al., 1975; Johansson et al., 1981; Poulat, 1992), and at early stages of development is found in the pancreas (Martino et al., 1978) and gastro-intestinal tract where it may be involved in the regulation of gastro-intestinal secretion and motility (Morley et al., 1977). TRH levels in these regions are high at neonatal stages of development and decline to low levels in the adult (Morley et al., 1979; Kawano et al., 1983). TRH is also present in the retina, where it appears to be regulated by environmental lighting (Schaeffer et al., 1977), and in the reproductive tissues of the male rat including testis, where it may play an autocrine/paracrine role in the regulation of testosterone secretion and spermatogenesis (Feng et al., 1993a) and prostate (Pekary et al., 1980). TRH has also been localised to human placenta (Shambaugh et al., 1979), see Chpt. 5. The recent identification of TRH-like peptides raises questions as to whether the initial experiments on TRH localisation identified authentic TRH or cross-reacted with TRH-like peptides (Fuse et al., 1990; Gkonos et al., 1993).

2.3.8 TRH as a neuromodulator/neurotransmitter

The widespread distribution of TRH, especially in the CNS, argues for a role for TRH as a neuromodulator or neurotransmitter (Sharif, 1985) (Table 2.1). The importance of TRH in the CNS is suported by its involvement (together with other CNS peptides) in the control of respiration, temperature regulation, hypertension, anorexia, locomotion and colonic motility following infusion of TRH into the CNS (Jackson, 1982; Scanlon & Hall, 1989). Evidence has indicated that TRH directly affects the electrical activity of single neurons and also the increased stimulation and release of neurotransmitters such as noradrenaline, dopamine, serotonin and acetylcholine (Winokur & Beckman, 1978; Kerwin & Pycock, 1979). TRH has been shown to co-exist with serotonin and substance P in the neuronal projections from the medulla oblongata to

Site of Action	Effect	Mechanism
Pituitary thyrotrophs	Stimulation of TSH release	Releasing hormone
lactotrophs	Stimulation of PRL release	
corticotrophs	Paradoxical stimulation of ACTH release (Cushing's disease)	
somatotrophs	Paradoxical stimulation of GH release (acromegaly)	
CNS olfactory system septal area amygdaloid complex cerebral cortex hypothalamus hippocampus basal ganglia motor nuclei of cranial nerves in brainstem spinal cord	-Alters firing rate of certain neurons -Increases noradrenalin and ACh turnover -Potentiates behavioural effects of dopamine and serotonin -Stimulates respiration -Suppresses feeding and drinking behaviour -Elevates blood pressure -May act as an anti-depressant -Induces hyperthermia -Antagonises narcosis induced by certain drugs -Antagonises effects of opiates without affecting analgesia	Neurotransmitter/ neuromodulator
Retina	TRH-like activity in retina regulated by environmental lighting - may be involved in photoreception	Neurotransmitter/ neuromodulator?
Gastro-intestinal tract	-Stimulation of gastro-intestinal motility -Increases gastric acid, pepsin and exocrine pancreatic secretion	Neurotransmitter/ neuromodulator?
Reproductive system Testis Prostate Placenta	-Testicular TRH may be an autocrine regulator of testosterone production and a paracrine regulator of spermatogenesis	Neurotransmitter/ neuromodulator?

Table 2.1

Multiple functions for TRH (adapted from Scanlon & Hall, 1989; Jackson, 1982).

the spinal cord (Johansson et al., 1981). Substance P apparently inhibits spinal TRH-R binding while co-depletion of serotonin and TRH results in up-regulation of spinal cord TRH-Rs (Ogawa et al., 1985). Some of the TRH effects are acute and transient, ie. neurotransmitter-type activity, whilst others are prolonged, suggesting a neuromodulatory role in interactions with other peptides or transmitters.

TRH can be localised to synaptosomal preparations and can be released from various brain regions by stimuli such as depolarisation (Maeda & Frohman, 1980). Specific high affinity TRH-Rs have also been found in various regions of the CNS using radio-labelled ligand-binding techniques and *in situ* hybridisation (Ogawa et al., 1981; Ogawa et al., 1985; Eymin et al., 1993; Zabavnik et al., 1993).

2.3.9 Clinical effects of TRH in the CNS

High levels of TRH have been observed in the basal ganglia of patients affected by Huntingdon's chorea and may therefore be implicated in the pathophysiology of this disease (Spindel et al., 1980). TRH may also be involved in modulating excitatory processes in epilepsy (Kubek et al., 1993).

Despite the fact that TRH crosses the blood-brain barrier poorly, it has been shown to reverse narcotic depression induced by barbiturates and ethanol in the rat, however the beneficial effects of TRH on such depression is controversial (Evans et al., 1975; Jackson, 1982; Scanlon & Hall, 1989). TRH has been shown to antagonise the hypothermic and cataleptic effects of opiates without affecting the analgesic effects they produce (Holaday et al., 1983; Metcalf & Dettmar, 1981). Therapeutic effects of TRH have been observed in various forms of shock in which opioid antagonists such as naloxone are traditionally used (Holaday & d'Amato, 1981). TRH was found to maintain blood pressure after haemorrhagic and endotoxic shock in the rat, and can also improve recovery and survival after spinal trauma injury in cats (Faden et al., 1981). TRH appears to mediate pain perception in complex interactions with substance P, enkephalin, oxytocin, vasopressin and angiotensin, all of which co-exist in the spinal cord.

TRH has been shown to stimulate increased locomotor activity, possibly by stimulating the release of dopamine from the nucleus

accumbens (Kerwin & Pycock, 1979; Collu et al., 1992) which has encouraged research into effects of TRH on motor function in patients suffering from spinocerebellar degeneration (Sobue et al., 1980) and amyotrophic lateral sclerosis (Engel et al., 1983).

2.3.10 Summary

The role for TRH as a hypothalamic releasing hormone is only one aspect of its diverse biological functions, although it is the only role that has been extensively characterised. The distribution and neuropharmacological effects of TRH support a neurotransmitter or neuromodulatory role for TRH in the CNS (Table 2.1). The involvement of TRH in the gastro-intestinal tract, reproductive tract and retina is even less well understood and will be the focus of much attention in the future. TRH appears to be an almost ubiquitous transmitter engaged in complex interactions with many other transmitters and peptides to regulate the normal functioning of the body. Using TRH as a therapeutic agent may disturb systems other than the particular one being treated. The detailed study of TRH-Rs in the pituitary, CNS and other locations will help to design and develop specific, non-degradable TRH analogues that can penetrate the blood-brain barrier and interact with a specfic receptor in a particular location (see Chpts. 4, 5 and 6).

2.4 Dopamine

The catecholamines, adrenaline (epinephrine), noradrenaline (norepinephrine) and dopamine (DA) are an important class of neurotransmitters involved in synaptic transmission in the central nervous system and are synthesised from the amino acid phenylalanine as shown in Fig. 2.6. Dopamine is the predominant catecholamine neurotransmitter in the mammalian brain (Smith, 1989) and it acts as a neurohormone in the anterior pituitary gland.

2.4.1 Dopamine as a neurotransmitter in the CNS

The dopaminergic system comprises three main neuronal pathways:- the nigrostriatal, mesocorticolimbic and tuberoinfundibular pathways. (Fig. 2.7) The nigrostriatal pathway contains neurons of the substantia nigra

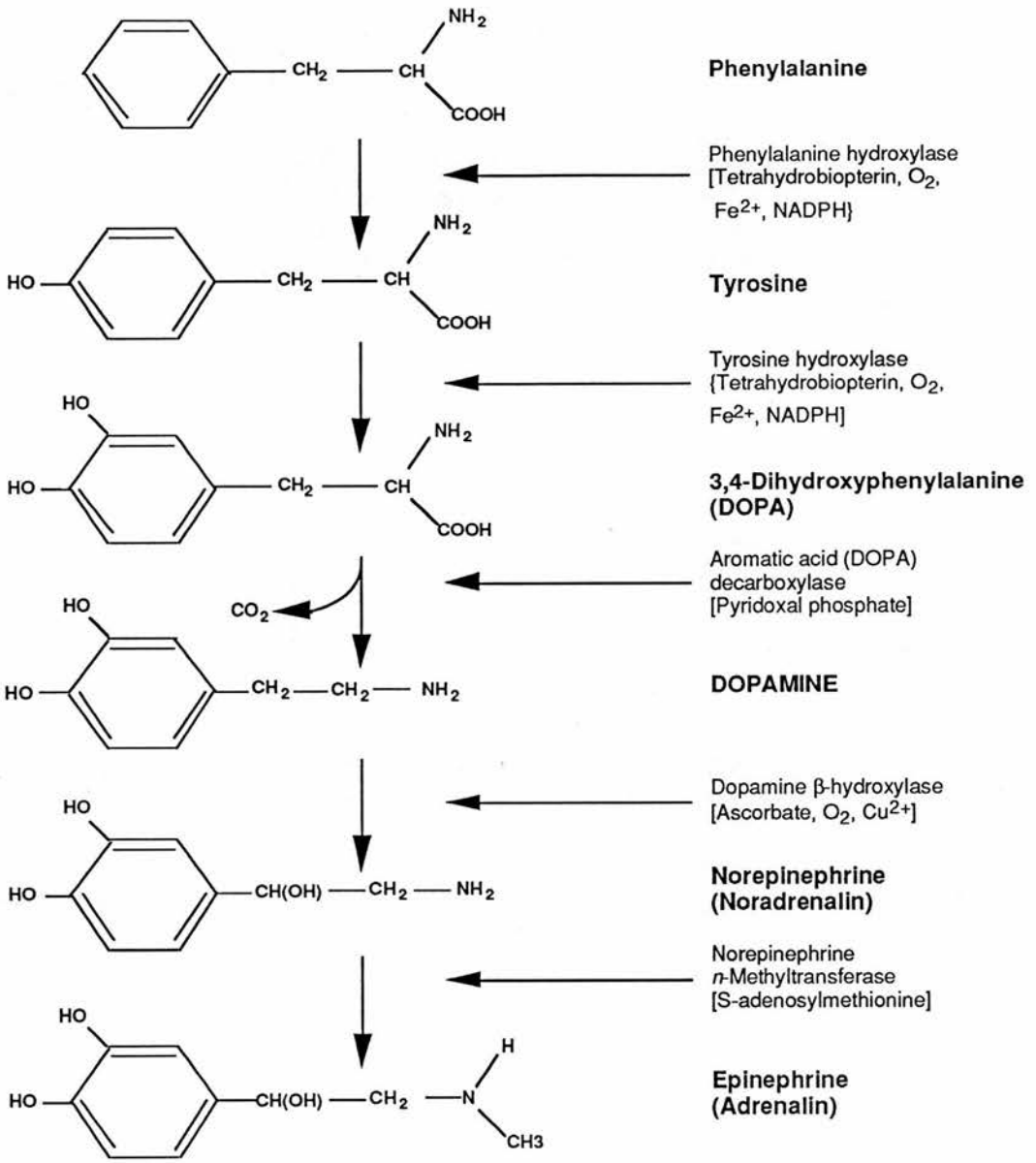


Fig. 2.6

Synthesis of the catecholamines, dopamine, noradrenaline and adrenaline (from Smith, 1989).

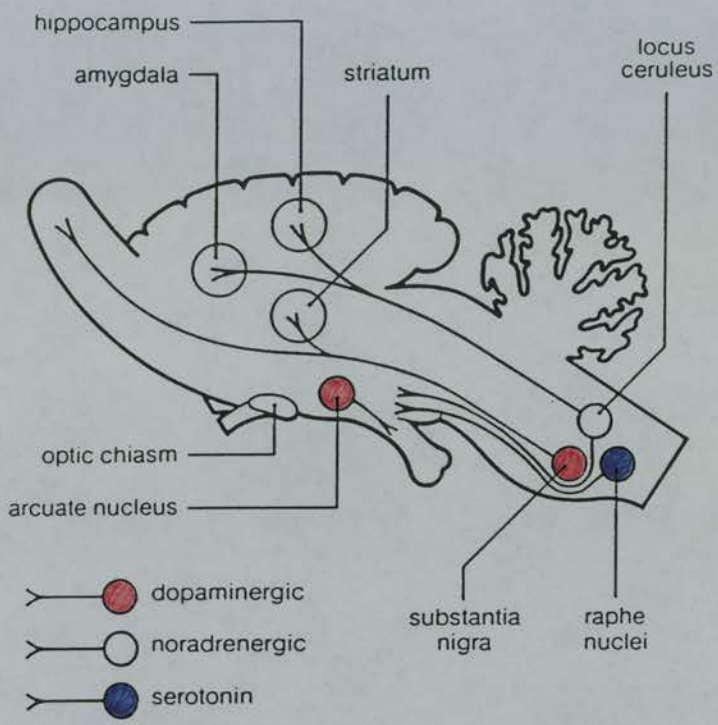


Fig. 2.7

Diagram showing the ascending bioaminergic tracts involved in hypothalamic-pituitary function in the rat. Dopaminergic fibres comprise one group whose origin is in the *substantia nigra* in the midbrain. An intrinsic tuberoinfundibular system is responsible for dopamine secretion into the hypophyseal-portal system which regulates prolactin secretion. Not shown is the mesolimbic system which innervates the visceral brain. Ascending fibres from the *locus ceruleus* bring noradrenergic influences into the hypothalamus, and fibres from the raphe nuclei carry serotonergic signals to the hypothalamus and elsewhere. All these neurotransmitter pathways are involved in the regulation of the anterior and posterior pituitary, in addition to other important visceral and behavioural effects (from Reichlin, 1987).

which synthesise DA and neurons of the striatum which respond to it. Degeneration of these neurons results in Parkinson's disease, thus this pathway appears to control normal movement (Lee et al., 1978). The mesocorticolimbic pathway consists of neurons of the central tegmental area which connect with those of the limbic fore-brain and is thought to be involved in controlling emotions. Breakdown of this system may contribute to schizophrenia and is the site of action of neuroleptic drugs (Seeman, 1987). The tuberoinfundibular neurons originate in the hypothalamus and DA is secreted by these neurons (known as the TIDA system) into the portal blood and is transported to the pituitary where its principle action is to inhibit prolactin secretion and thus regulate lactation and fertility (Birge et al., 1970). Dopamine also acts to inhibit TSH secretion from the anterior pituitary (Scanlon et al., 1979; Besses et al., 1975).

Clinical pathologies such as Parkinson's disease and schizophrenia were the main reasons for stimulating research into the dopaminergic system, resulting in the elucidation of the different pathways and the family of receptors involved (Kebabian & Calne, 1979; Civelli et al., 1991; Civelli et al., 1993).

2.4.2 Dopamine receptors in the CNS

Unlike the TRH system, where the identification and analysis of specific receptors and receptor subtypes is just beginning, the existence of a family of DA receptors (DA-Rs) has been known for some time. Certain criteria were laid down for the classification of receptors for a particular transmitter. Firstly, the endogenous ligand must be identified. Since 1966, Parkinson's patients had been treated with the amino acid L-dihydroxyphenylalanine (L-DOPA), a precursor in the synthesis of the catecholamines (Kebabian & Calne, 1979). This produced 'miracle cures' and identified a role for DA in controlling normal movement via a DA-R in the nigrostriatal region.

The discovery of antipsychotic drugs known as neuroleptics in 1952 (Seeman, 1987) stimulated extensive research into their mode of action. These drugs were used to relieve the symptoms of schizophrenia, but their sites of action were unknown. It was discovered that long term treatment with neuroleptics could produce effects similar to the movement

disorders found in Parkinson's disease, while high doses of DA (L-DOPA) used to treat Parkinson's patients could result in psychoses (such as hallucinations) common to schizophrenics. These observations pointed to the involvement of dopaminergic systems in the etiology of schizophrenia as well as Parkinson's disease, suggesting that the binding sites for neuroleptic drugs might be DA -Rs (Seeman, 1975; Seeman, 1977). It was also suggested that since separate neural pathways were involved in the two disorders, the DA-Rs involved in each pathway might also be subtly different.

2.4.3 Pharmacological classification of dopamine receptors

Once the endogenous ligand has been identified, receptors can be classified pharmacologically, based on the action of drugs that selectively mimic (agonists) or inhibit (antagonists) the effects of the endogenous ligand. The receptor should respond to minute quantities of a particular drug in a specific manner.

DA-Rs were initially characterised according to their responses to various drugs including DA itself, and agonists like apomorphine and the amphetamines which, in large doses, produced schizophrenic symptoms. Neuroleptics such as the phenothiazines and butyrophenones alleviated the symptoms of schizophrenia and were thought to block DA-Rs (Snyder et al., 1974).

To explain the varying potencies of the different drugs, it was initially thought that there might be two DA-Rs located on different cell types. Direct binding to DA-Rs in the brain was achieved with radio-labelled ligands. [^{3}H]DA itself, and [^{3}H]haloperidol (a member of the butyrophenone antagonists) were shown to bind with high affinity and selectivity to synaptic DA-Rs in membrane preparations of the calf caudate. The ability of various antipsychotic agents in displacing [^{3}H]DA and [^{3}H]haloperidol binding correlated well with the clinical potencies of these drugs (Creese et al., 1975; Seeman, 1975; Burt et al., 1975).

The similar regional distribution of [^{3}H]DA and [^{3}H]haloperidol binding suggested that one DA-R existed in two different states with selective high affinities for agonists and antagonists, rather than there being two separate receptors. The demonstration that glycine receptors

had two such binding sites, supported the possibility (Young & Snyder, 1974), although this theory was later disproved.

2.4.4 Dopamine-sensitive adenylate cyclase

The demonstration of a DA-sensitive adenylate cyclase in the superior cervical sympathetic ganglion, substantia nigra and the retina provided an *in vitro* model for studying the properties of the DA-R (Kebabian & Greenwood, 1971; Iversen, 1975; Clement-Cormier et al., 1974, Kebabian et al., 1972). Dopaminergic stimulation of cAMP formation in bovine parathyroid cells led to the release of parathyroid hormone (Brown et al., 1977), further suggesting a role for the adenylate cyclase-cAMP signal transduction system in the propagation of a physiological response to DA.

Not all DA-Rs, however, shared the same properties, casting doubt on the 'one receptor, two binding states' model (Kebabian & Calne, 1979). Discrepancies arose from the action of the butyrophenones which did not correlate with their ability to inhibit the DA-sensitive adenylate cyclase. These drugs were potent clinically, but were weak inhibitors of adenylate cyclase (Karobath & Leitich, 1974; Roufogalis et al., 1976; Seeman, 1977).

2.4.5 More than one dopamine receptor?

It was becoming apparent that there might be more than one DA-R, although confusion still existed due to the overlapping dopaminergic binding sites in the brain (Creese et al., 1975). Direct evidence for at least two classes of DA-R came from results showing that most of the striatal ^{3}[H]haloperidol binding sites in the rat were physically separate from the DA-sensitive adenylate cyclase binding sites (Schwarz et al., 1978).

2.4.6 Dopamine and the hypothalamic-pituitary axis

The primary sites of DA action were thought to occur in the CNS until its role as a neurohormone, regulating pituitary hormonal secretion, became appreciated. Catecholamines were found to inhibit the release of prolactin from the anterior pituitary (Steiner et al., 1970). Further studies with selective catecholamine agonists and antagonists on rat anterior pituitary glands suggested that DA was the major physiological inhibitor of prolactin secretion, acting directly on the anterior pituitary gland via

specific DA-Rs (MacLeod & Lehmeyer, 1974; Yamauchi et al., 1977), see section 2.5.1. The presence of DA in the anterior pituitary gland (Saavedra et al.,1975) and in the hypophyseal portal circulation (Ben-Jonathan et al., 1977) supported this theory. DA is secreted into the portal vessels of the pituitary gland by the neurons of the TIDA system, whose cell bodies are located in the arcuate nucleus (Fig. 2.7). DA also inhibits the secretion of TSH from thyrotrophs in the anterior pituitary (Fig. 2.2). The major inhibition of TSH is due to feedback of the thyroid hormones (section 2.3.3), but DA does seem to be involved, although in a more modulatory role (Besses et al., 1975; Scanlon et al., 1979).

2.4.7 Dopamine binding sites in the pituitary

The use of the ergot alkaloid [^{3}H]dihydroergocryptine (a potent DA agonist) to study binding sites in bovine pituitary membranes indictated that binding was associated with mammotrophs (lactotrophs) and that these DA sites modulated prolactin secretion (Caron et al., 1978). This represented the first direct study of DA binding sites in the anterior pituitary and the first correlation with binding of a dopaminergic ligand to a DA-regulated physiological process.

The use of selective radio-labelled antagonists confirmed the presence of these regulatory DA binding sites (Calabro & MacLeod, 1978) and suggested a difference between brain and pituitary DA-Rs. In the anterior pituitary, apomorphine acted as a DA agonist (Caron et al., 1978), however in tissues where DA-Rs were linked to adenylate cyclase, apomorphine acted as a DA antagonist. Also, brain preparations contained DA-sensitive adenylate cyclase, whilst there was no catecholamine activation of this enzyme in the anterior pituitary (Steiner et al., 1970).

DA was shown to inhibit basal levels of adenylate cyclase in homogenates of human prolactin-secreting adenomas and the DA-Rs mediating this inhibition had the same pharmacological properties as those regulating prolactin secretion (de Camilli & Macconi, 1979). Studies with various DA agonists and antagonists in rat anterior pituitary cells confirmed that the pituitary DA-R involved in the inhibition of prolactin secretion was negatively coupled to adenylate cyclase (Enjalbert &

Bockbaert, 1983). The action of dopamine via the pituitary DA-R in the anterior pituitary gland is discussed in more detail in section 2.5.1.

2.4.8 Two classes of dopamine receptor: D1 and D2

In 1979 DA-Rs were placed in two distinct categories based on the accumulation of physiological, pharmacological and anatomical data and were called D1-like and D2-like receptors (Kebabian & Calne, 1979). Briefly, D1-like receptors were described as being linked to the stimulation of adenylate cyclase and were not found in the anterior pituitary, while D2-like receptors inhibited adenylate cyclase, had a high affinity for neuroleptics, especially the butyrophenones, and a lower affinity for DA agonists. Both D1 and D2-Rs existed in the brain, often with overlapping distributions and both classes of receptor were found to occur both pre-synaptically (autoreceptors) and post-synaptically in the dopaminergic neurons (Table 2.2).

For just over a decade, this two-receptor classification has explained most of the observed effects of DA in the different dopaminergic pathways. The existence of other DA-Rs was guessed at (Enjalbert & Bockaert, 1983), but remained elusive, masked by the effects of the D1 and D2 receptors.

Criteria	**D1-like**	**D2-like**
Adenylate cyclase coupling	Stimulates	Inhibits
Distribution of proto-type receptor	Parathyroid	Lactotrophs of anterior pituitary
Dopamine	Agonist (μM potency)	Agonist (nM potency)
Ergot derivatives eg. bromocryptine	Potent antagonist (nM potency) Weak agonist (μM potency)	Agonist (nM potency)
Selective antagonist	None known in 1979	Metoclopramide sulpiride
Radio-labelled ligand	*cis*-flupenthixol	Dihydroergocryptine

Table 2.2

D1/D2 classification of Dopamine Receptors (from Kebabian & Calne, 1979).

2.4.9 Multiple dopamine receptor subtypes

The discovery that DA-Rs were coupled to G-proteins (Vallar & Melddesi, 1989) eventually led to their molecular characterisation. The existence of a superfamily of similar membrane G-protein-coupled receptors (GPRs) was used to clone new receptors by homology screening, using nucleic acid probes based on observed sequence similiarities. Thus prior knowledge of peptide sequence or even biological activity was unnecessary when cloning novel receptors (Civelli et al., 1993 for review).

The rat D2-R was cloned using the hamster β-adrenergic receptor sequence (a member of the GPR family) as a probe for homology screening (Bunzow et al., 1988). The cloning and characterisation of the mouse gene for this receptor is discussed in detail in Chpt. 7. The D1-R was cloned from rat striatum using the polymerase chain reaction (PCR) (Zhou et al., 1990; Monsma et al., 1990) and also by homology screening (Dearry et al., 1990; Sunahara et al., 1990). These putative D1-Rs were expressed in transfected cell lines and were shown to stimulate adenylate cyclase confirming their identity.

Since then, three other DA receptors have been cloned. The D3-R (Sokoloff et al., 1990) and D4-R (Van Tol et al., 1991) are most closely related to the D2 receptor, but each has a distinct pharmacological profile. The D5-R (Sunahara et al., 1991, Grandy et al., 1991; Tiberi et al., 1991) is similar to the D1-R in that it stimulates adenylate cyclase, however, it has a higher affinity for dopamine.

2.4.10 Even more dopamine receptors?

There is evidence for yet more DA-Rs. A D2-like receptor that binds spiperone (a DA antagonist) but has a different nucleotide sequence and which does not appear to be linked to a G-protein, has been reported (Todd et al., 1989). D1-like receptors have been detected in renal tissue (Goldberg et al., 1978; Felder et al., 1989) and are linked to the activation of phospholipase C and the phosphoinositide (PI) second messenger pathway, rather than adenylate cyclase. The cloned D1-R was not expressed in renal tissue (Dearry et al., 1990; Zhou et al., 1990). A D1-like receptor, but of a different size, coupling to the PI pathway has also been isolated from rat striatum mRNA (Mahan et al., 1990).

2.4.11 Summary

Dopamine is a major neurotransmitter in the CNS where it is involved in regulating emotion and movement. Disruptions in these dopaminergic pathways (possibly at the receptor level in some cases) result in common disorders such as Parkinson's disease, schizophrenia and susceptibility to alcohol and drug abuse. DA also acts as a neurohormone in the hypothalamus, inhibiting the release of prolactin and TSH from the anterior pituitary. The existence of multiple DA-R subtypes has affected preconceptions of the dopaminergic system. Overactive D2-Rs were traditionally thought to be responsible for schizophrenia, but recent evidence has implicated the D4-R subtype (Seeman et al., 1993; Iversen, 1993). Characterisation of the DA-R subtypes and their genes is critical for designing selective agonists/antagonists for specific DA receptors and this molecular information will also be useful in linking specific DA receptors to particular disease states. Chpt. 7 reports the cloning and characteristion of the mouse D2-R gene.

2.5 Regulation of prolactin release by dopamine and TRH

The regulation of prolactin secretion from lactotrophs in the anterior pituitary appears to involve complex interactions between multiple intracellular signalling pathways linked to DA, TRH and VIP (Fig. 2.2 and Fig. 2.8).

2.5.1 Prolactin inhibitory factors

The major regulatory role of the hypothalamus is inhibitory with respect to prolactin secretion. It has long been known that dopamine inhibits adenylate cyclase via a specific receptor on lactotrophs (section 2.6.9) and that this is related to the inhibition of prolactin release (de Camilli et al., 1979; Schettini et al., 1983). It is now clear that it is the withdrawal of dopamine that results in the secretion of prolactin from anterior pituitary lactotrophs (Fig. 2.11). The dissociation of DA from its lactotroph D2-R releases adenylate cyclase which can then activate the cAMP pathway. This process may potentiate the action of prolactin releasing factors (PRFs) (de la Escalera & Weiner, 1992) see Fig. 2.8 and 2.11. Other

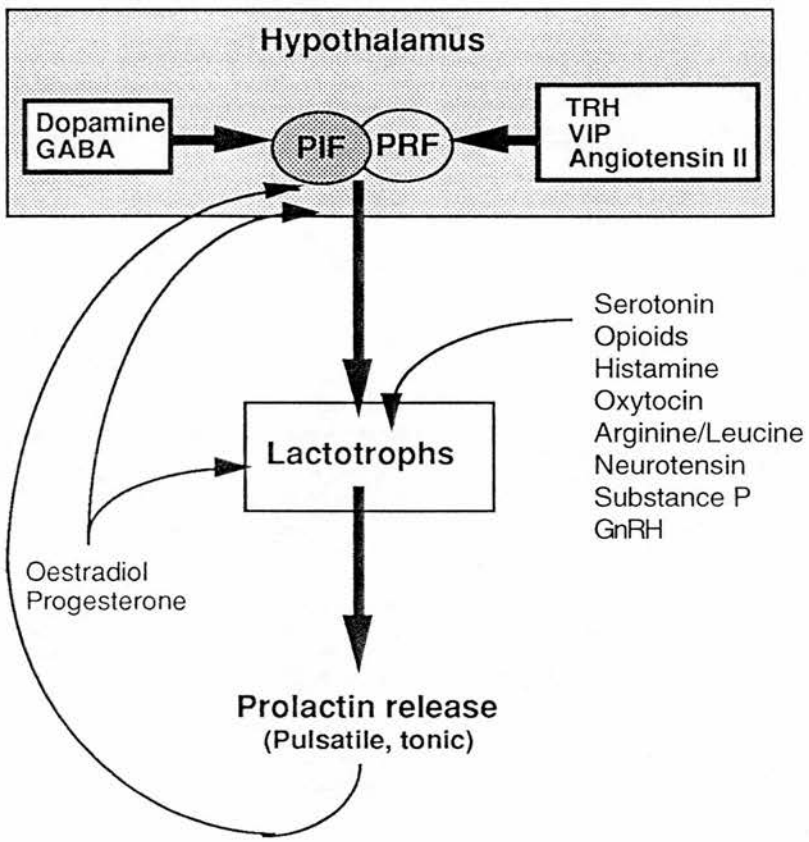


Fig. 2.8

Diagrammatic representation of neuroendocrine regulation of prolactin secretion. A variety of prolactin-inhibiting factors (PIFs) and prolactin-releasing factors (PRFs) as well as central and peripheral modulators are depicted. Several peptides regulate prolactin secretion within the pituitary gland by means of autocrine and paracrine mechanisms. Peripheral hormones, particularly oestradiol and progesterone, exert their influence through both hypothalamic and pituitary sites (Adapted from Yen, 1991).

prolactin-inhibiting factors (PIFs) include α-melanocyte-stimulating hormone and possibly GABA, which may act directly, but is less potent than DA (Yen, 1991b).

2.5.2 Prolactin releasing factors

Increases in prolactin secretion without measureable decreases in dopamine levels (Neill et al., 1981), together with an acute prolactin release observed under maximal inhibition by dopamine (Clemens et al., 1983) suggested the existence of one, or more, prolactin-releasing factors (PRFs). The time-course of circulating levels of prolactin in response to TRH, its dose-reponse characteristics and its suppressibility by T_4 pre-treatment were similar to those for TSH, suggesting that TRH might play a role in stimulating prolactin secretion (Leong et al., 1983) (Fig. 2.8). TRH has been shown to be a potent stimulator of prolactin release *in vitro* from dispersed anterior pituitary cells in culture, and the administration of TRH antiserum results in a decrease in both prolactin and TSH in rats (Koch et al., 1977). Specific TRH-Rs have been found on lactotrophs (Labrie et al., 1972; Hinkle & Tashjian, 1973) and TRH has been shown to stimulate prolactin gene transcription within minutes, as well as stimulating an acute release of prolactin (Gershengorn et al., 1984) via the Ca^{2+}/protein kinase C pathway (see section 2.6.8 and Fig. 2.11).

Evidence against a primary role for TRH in regulating prolactin comes from the observation that TRH antiserum did not prevent the release of prolactin in the ewe (Fraser & McNeilly, 1982) or in the rat (Harris et al., 1978). Circadian rhythms of prolactin and TSH are dissociated in humans (Harris et al., 1978), further supporting only a modulatory role for TRH. The hypothalamic hormone VIP, positively coupled to adenylate cyclase, also stimulates the release of prolactin in an autocrine manner (Nagy et al., 1988).

2.5.3 Modulation of prolactin secretion

The effects of TRH on prolactin secretion are known to be modulated by various peripheral hormones and neurotransmitters. In cases of hypothyroidism, low levels of T_3/T_4 stimulate prolactin release and in hyperthyroidism, high levels of thyroid hormones inhibit prolactin release (Snyder et al., 1973). Oestrogens are known to potentiate TRH-induced

prolactin release by interfering with the coupling of the dopamine D2-R to its second messenger system and by up-regulating the numbers of TRH-Rs on lactotrophs (Raymond et al., 1978; Munemura et al., 1989). Oxytocin, angiotensin II, serotonin, endogenous opioids, histamine and neurotensin have also been implicated in modulating the response of prolactin to TRH (Yen, 1991).

Since prolactin release is not regulated by feedback from its target organ (the mammary gland), prolactin itself may regulate secretion of PIFs and PRFs by a short-loop feedback mechanism. This is supported by the fact that central injection of prolactin results in an increase in both DA turnover in the median eminence and in the DA concentration in the portal blood (Gudelsky & Porter, 1980), and by the presence of prolactin-binding sites in the median eminence (Barton et al., 1989).

2.6 G-protein-coupled receptors

The dopamine D2-R and the TRH-R are both members of the G-protein-coupled receptor (GPR) superfamily. The D2-R is negatively coupled to the adenylate cyclase-cAMP second messenger pathway via the G-protein, G_i, while the TRH-R stimulates the phospholipase C-inositol phosphate pathway via G_q and/or G_{11}. This section describes general structural and functional features common to the members of this receptor family.

The concept of molecules able to recognise specific stimuli and transduce the signal to the intracellular environment was proposed by Langley in 1905 to explain the action of curare on skeletal muscle (Ahlquist, 1948). In 1906, Henry Dale suggested a receptive mechanism for adrenaline to explain the fact that ergot alkaloids prevented only the excitatory actions of adrenaline and had no effect on its inhibitory actions. The adrenotropic receptors were therefore considered to belong to two classes, those that generally resulted in excitatory functions, the alpha receptors, and those generally responsible for inhibition of effector cells, the beta-adrenergic receptors (Alquist, 1948; Brazier, 1959).

2.6.1 Receptors and Adenylate Cyclase

One of the early concepts of a cell-surface receptor was proposed by Robison and coworkers (Robison et al., 1967), who suggested that a

specific binding site might involve a single 'receptor' protein extending from one side of the cell membrane to the other. Adenylate cyclase, an enzyme known to reside in the cell membrane (Davoren & Sutherland, 1963) and to respond to stimulation by adrenaline (Øye & Sutherland, 1966) was suggested for the role of receptor. The enzyme was thought to have two subunits, one regulatory and facing the extracellular fluid (the two classes of adrenergic receptor were initially thought to be opposing regulatory elements of the adenylate cyclase receptor), and one catalytic and facing the cytoplasm, involved in converting ATP to adenosine 3',5'-monophosphate (cAMP). cAMP acted as a second messenger, translating the extra-cellular signals into a biological response within the cell.

The study of the adrenergic receptor-adenylate cyclase response system in erythrocytes became the focus for elucidating the mechanisms of transmembrane signalling as erythrocytes were a convenient source of receptor and the adenylate cyclase system was well known (Hoffman & Lefkowitz, 1980; Lefkowitz et al., 1983; Levitski, 1988).

2.6.2 Pharmacological ligand binding assays

Direct detection of receptors only became possible in the 1970s when pharmacological binding assays were developed (Hoffman & Lefkowitz, 1980). These assays exploited the use of radio-labelled agonists and antagonists for particular chemical transmitters and thus it was discovered that the beta-adrenergic receptor (βAR) was an independent unit that could be transferred from one adenylate cyclase system to another (Orly et al., 1976). Limbird and Lefkowitz (Limbird & Lefkowitz, 1977) used the frog erythrocyte system to confirm that the functions of βAR binding and adenylate cyclase activity were carried out by separate molecular entities.

Binding studies, supported by biochemical data (Hoffman & Lefkowitz, 1980; Lefkowitz et al., 1983; Levitski, 1988) confirmed the existence of the two major classes of adrenergic receptor which had been known since 1947 (Alquist, 1948) and allowed their further division into subclasses termed alpha $(\alpha)_1$ and α_2, and β_1 and β_2. Both βARs stimulated adenylate cyclase, but had different affinities for adrenaline and noradrenaline (norepinephrine). The α_2AR mainly inhibited adenylate cyclase, but α_1ARs did not seem to be linked to the adenylate cyclase

system. Thus the concept of multiple receptor subtypes for an endogenous ligand, exerting multiple cellular responses was beginning to capture attention.

2.6.3 Receptor purification

The development of affinity chromatography columns in the 1970s (Lefkowtiz et al., 1983) allowed the purification of minute amounts of receptor proteins. The β_1AR was purified from turkey erythrocytes (Vauquelin et al., 1977; Vauquelin et al., 1979) and the muscarinic cholinergic receptor from calf forebrain (André et al., 1983). The purified receptors were shown to have all the properties of their endogenous membrane-bound counterparts using ligand-binding assays, and reconstitution experiments in lipid vesicles (Lefkowitz et al., 1983).

2.6.4 G-protein-coupled receptors

Many hormone/neurotransmitter receptors respond to ligand binding by activating an enzyme such as adenylate cyclase or phospholipase C. In 1971, Martin Rodbell (Rodbell, 1980) discovered that the nucleotide, guanine triphosphate (GTP), was essential for the activation of adenylate cyclase and that its action was enhanced by hormones. This led to Rodbell's 'G-protein theory' in which he suggested that a nucleotide regulatory unit (G-protein) with site(s) for binding GTP was located at the inner face of the plasma membrane and was responsible for mediating the effects of GTP and hormones on adenylate cyclase, the catalytic component of the system.

2.6.5 Heterotrimeric G-proteins

The first G-protein to be identified and sequenced was G_s, an adenylate cyclase-stimulating GTP-binding protein, involved in mediating the effects of adrenaline on adenylate cylcase (Gilman, 1987; Birnbaumer, 1990). G_s consists of three tightly associated subunits, α, β and γ in a 1:1:1 ratio and was most similar to transducin (Stryer, 1986), a GTP-binding protein in the visual system, coupled to the photoreceptor rhodopsin, another member of the GPR family.

Reconstitution experiments in which the purified components of the proposed signalling system, ie. receptor, G-protein and adenylate

cyclase, were combined in phospholipid vesicles, established that receptors could interact directly with GTP-binding proteins. (Lefkowitz et al, 1983). These experiments also showed that the ligand binding and G-protein coupling sites of a receptor existed in a single molecule (Asano et al., 1984).

G-proteins were defined according to their sensitivity to ADP-ribosylation of the α-subunit by pertussus toxin, or cholera toxin (Gilman, 1987; Neer & Clapham, 1988; Birnbaumer, 1990; Simon et al., 1991; Bourne et al., 1991). Initially, the cholera toxin substrates were thought only to stimulate adenylate cyclase, and the pertussis toxin substrates to inhibit the enzyme, and the convention has been to name the G-protein after its α-subunit, for example $G\alpha_s$ and $G\alpha_i$ respectively. The rhodopsin G-protein, transducin, is a substrate for both toxins (Neer & Clapham, 1988). It is now known that G-proteins can also couple receptors to other second messenger pathways, such as the PI pathway and can regulate ion-channels (Kuo & Bean, 1993).

G-proteins, like the receptors, also belong to a large superfamily of related nucleotide-binding proteins. More than thirty different G-protein α-subunits have been cloned and are separated into four classes based on amino acid similarity: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ (Simon et al., 1991). Subunit diversity also exists amongst the β and γ subunits (Birnbaumer, 1990).

2.6.6 The G-protein cycle

The agonist-stimulated receptor has an increased ability to bind to the inactive $G\alpha\beta\gamma$ heterotrimer (by a mechanism not yet understood) and this interaction leads to the release of GDP bound to the α-subunit (Fig. 2.9). GTP has a higher affinity than GDP for the 'empty' Gα subunit and the binding of GTP mediates a conformational change in the α-subunit which alters its affinity for binding other macromolecules, resulting in its activation. The activated α-subunit then associates with an effector molecule such as a phosphodiesterase, adenylate cyclase, phospholipase C, phospholipase A2 or an ion channel (Simon et al., 1991). The $\alpha\beta\gamma$ complex is transient since the binding of GTP results in the dissociation of the GTP-α-subunit complex from the βγ dimer. The intrinsic GTPase activity

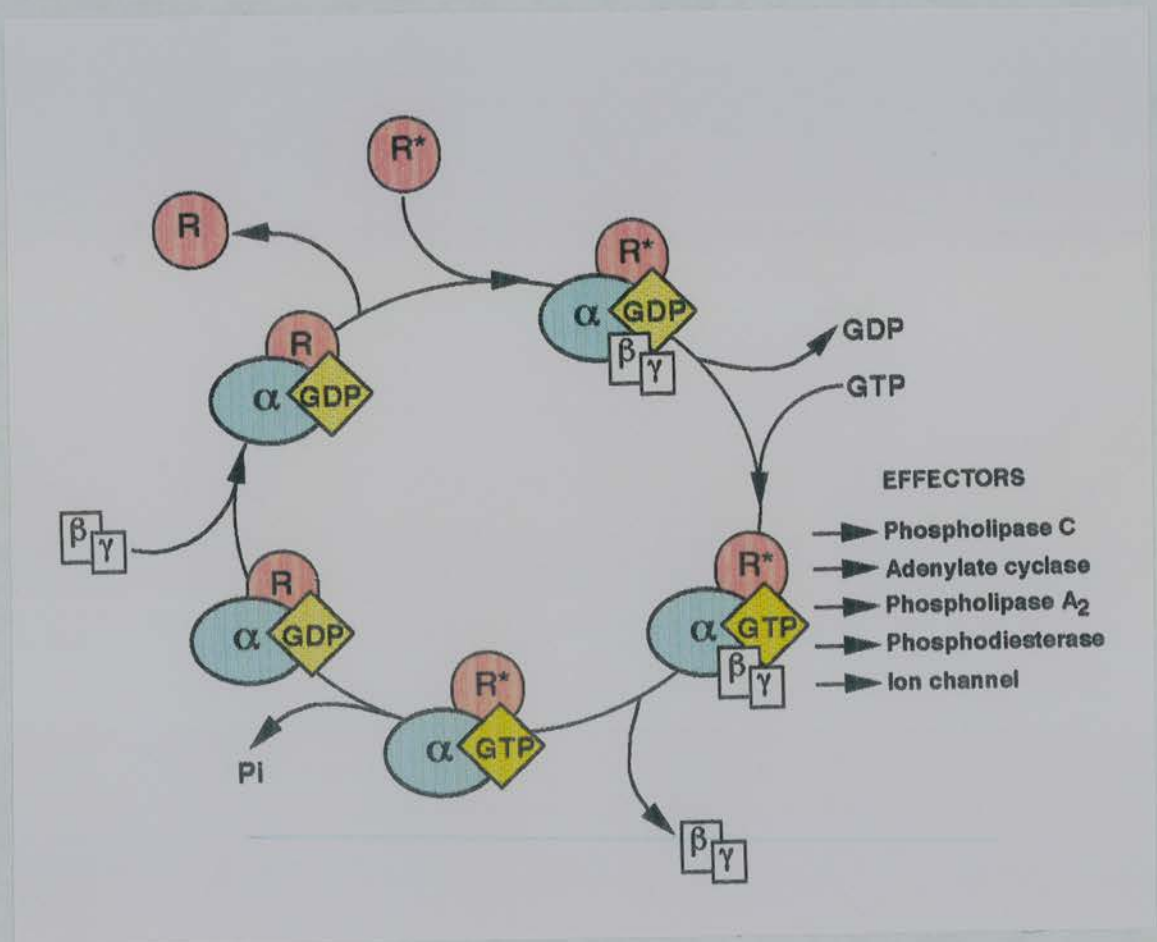


Fig. 2.9

The G-protein cycle. A ligand-bound receptor (R*) catalyses the exchange of bound GDP for GTP on the G-protein, which in turn, interacts with an effector protein to produce an intracellular signal. The activated state of the G-protein is terminated by an intrinsic GTPase activity, resulting in the hydrolysis of bound GTP to GDP and the dissociation of the α-subunit from the βγ dimer.

of the α-subunit hydrolyses bound GTP to GDP, and the GDP-α-subunit can once again reassociate with the βγ dimer. The receptor uncouples from the inactive αβγ protein and is ready to activate the next G-protein (Gilman, 1987; Neer & Clapham, 1988; Birnbaumer, 1990; Simon et al., 1991; Bourne et al., 1991). G-proteins, therefore, function as molecular switches, being turned on by binding GTP and off by hydrolysing GTP to GDP. Until recently, the Gα-subunit alone was thought to activate effector molecules, but newer evidence has indicated that the βγ dimer can also regulate effector pathways, increasing the complexity of the cell's signalling system (Federman et al., 1992; Clapham & Neer, 1993).

2.6.7 GPRs and signal transduction

The heterotrimeric G-proteins activate various intracellular signal transduction pathways by their affinity for binding and activating enzymes such as phospholipase C or adenylate cyclase (Table 2.3).

G-protein	Effector
G_s, G_i	Adenylate cyclase regulation
G_t, G_{olf}	Sensory transduction
G_q, G_{11}	Phospholipase activation
G_o, G_s	Ion channel activation

Table 2.3 G-proteins coupling to second messenger pathways.

These enzymes generate regulatory molecules or 'second messengers' which can then effect physiological responses such as selective protein phosphorylation, gene transcription, reorganisation of the cytoskeleton, protein secretion and membrane depolarisation. The availability of multiple receptor subtypes, multiple G-proteins and multiple isoforms of the signal transduction enzymes, suggests a complex network of cellular interactions required to co-ordinate intracellular communication (Suh et al., 1988; Krupinski et al., 1989; Kuo & Bean, 1993). Recently, some receptors have been shown to activate both the adenylate cyclase and phospholipase C pathways *in vitro*, or to activate one and inhibit the other, and several models for multiple signalling pathways by GPRs have

been proposed (Fig. 2.10). It is becoming apparent, however, that the activation of particular signalling pathways by transfected receptors depends upon the environment provided by the particular cell-line used (Birnbaumer, 1990; Milligan, 1993).

2.6.8 Phospholipase enzymes and signal transduction

Stimulation of cell surface receptors, eg. TRH-Rs and activation of the appropriate G-protein (Table 2.3) results in the activation of a phospholipase enzyme (a minor constituent of the plasma membrane) which in turn catalyses the hydrolysis of a membrane-bound phospholipid (Fig. 2.11). This hydrolysis produces at least two second messenger molecules, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG remains in the plasma membrane and activates protein kinase C (PKC), while IP3 binds to intracellular vesicles that store calcium ions (Ca^{2+}). Calcium is released into the cytoplasm where it may bind calmodulin, a regulatory subunit for other enzymes such as protein kinases. Inositol phosphates can also promote an influx of extracellular Ca^{2+} through ion channels in the plasma membrane, in response to certain agonists.

There are multiple forms of inositol phosphates regulating Ca^{2+} influx into the cell as well as the mobilisation of the various intracellular Ca^{2+} pools. The inositol phosphate (IP) signalling system is complex and widespread allowing a cell to distinguish between different receptors involved in controlling short-term functions such as protein secretion in response to a hormonal stimulus, or long-term responses such as cell growth (Berridge & Irvine, 1989).

2.6.9 Adenylate cyclase and signal transduction

The adenylate cyclase signalling system has been the focus of attention for many years. Its second messenger molecule, cAMP is capable of regulating many different biochemical pathways and its discovery by Sutherland in the 1950s resulted in the Nobel Prize for physiology and medicine in 1971 (Bloom, 1981). Receptor stimulation of an appropriate G-protein by VIP or DA withdrawal, for example (Table 2.3) triggers the activation of adenylate cyclase within minutes, which catalyses the formation of cAMP from ATP (Fig. 2.11).

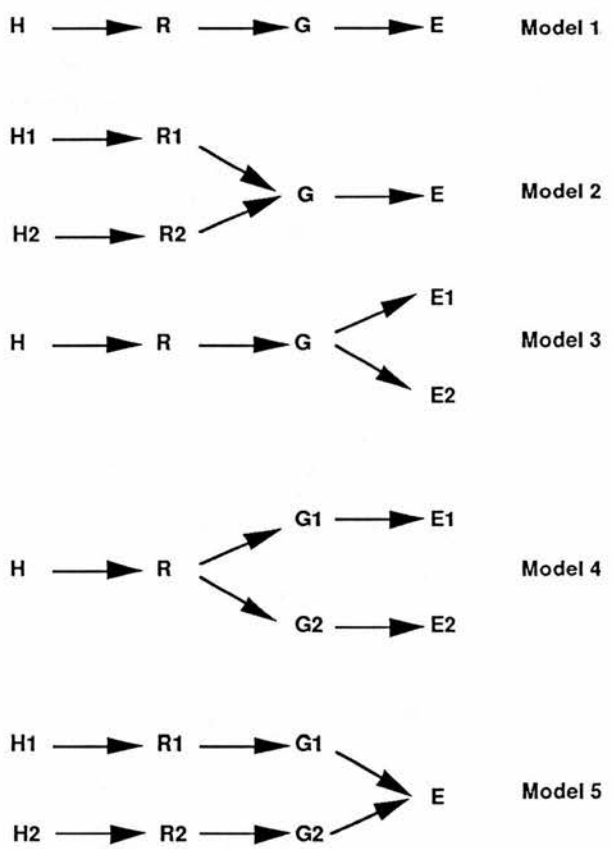


Fig. 2.10

Mechanisms for signal bifurcation by G-protein-linked receptors. Model 1: One receptor subtype interacts with one G-protein. Model 2: Different ligands activate their respective receptors which can couple to the same G-protein. Model 3: A single receptor interacts with a single G-protein, but signal bifurcation is achieved by regulation of different effectors by the G-protein α- and $\beta\gamma$-subunits. Model 4: A single receptor activates multiple G-proteins, each of which can regulate a separate effector. Model 5: Different G-proteins coupled to different receptor systems can activate the same effector (Adapted from Birnbaumer, 1990 and Milligan, 1993).

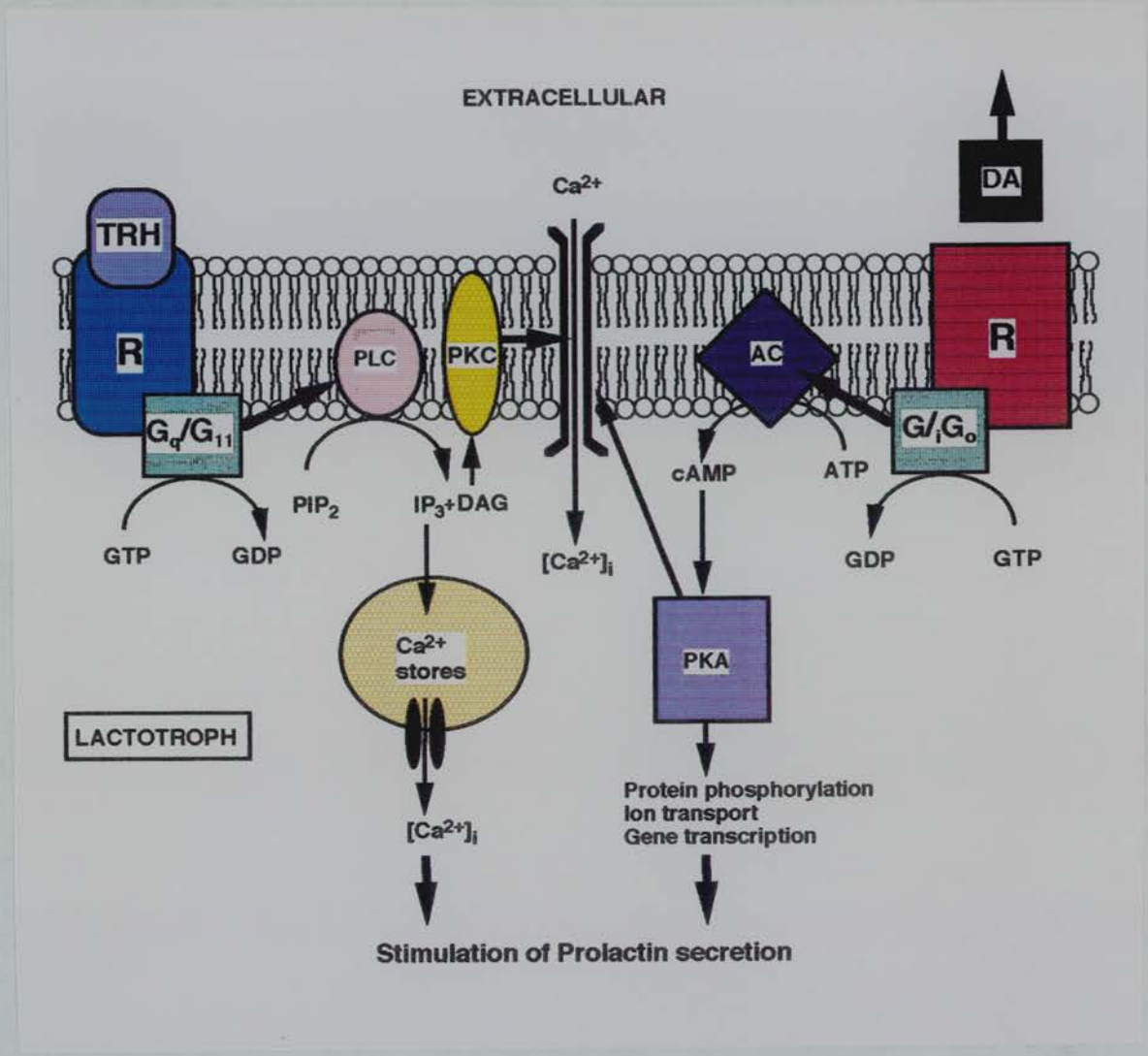


Fig. 2.11

Simplified diagram illustrating putative second messenger coupling pathways involved in the regulation of prolactin secretion. The stimulatory action of TRH may be potentiated by prior activation of the protein kinase A (PKA) pathway. Withdrawal of dopamine (DA) from its receptor (R) activates adenylate cyclase (AC) generating the second messenger cAMP. PKA pathway activation by the dissociation of DA from its receptor, or by the association of VIP with its receptor (not shown), has a prolonged effect, and may modulate the response to TRH by phosphorylating voltage-gated Ca^{2+} channels. Association of TRH with its receptor activates the phospholipase C (PLC) enzyme which generates two second messengers, diacylglycerol (DAG) activating protein kinase C (PKC) resulting in the phosphorylation of Ca^{2+} channels, and inositol 1,4,5-trisphosphate (IP_3) which effects the release of Ca^{2+} from intracellular stores.

Amplification of the original receptor stimulus is due to the slow GTP-hydrolysis step, which allows the production of approximately a hundred cAMP molecules before GTP decays to GDP and the ligand-receptor-G-protein-enzyme complex is inactivated. One receptor can activate approximately ten adenylate cyclase molecules, amplifying the original stimulus approximately a thousand times. cAMP exerts its effects on the intracellular environment through cAMP-dependent protein kinase A (Levitski, 1988).

2.6.10 Cloning of G-protein-coupled receptors

While the G-protein theory was being proposed, microsequencing of purified receptor proteins resulted in the production of oligonucleotides used to isolate receptor genes. Rhodopsin, a GPR in the visual system was cloned and sequenced in 1982 (Ovchinnikov, 1982) and the βAR receptor was cloned and sequenced in 1986 from hamster (Dixon et al., 1986). Surprisingly, hydrophobicity analysis indicated that both receptors appeared to have a similar structure to bacteriorhodpsin, a non-G-protein-coupled, light-driven proton pump isolated from *Halobacterium halobium*. Amino acid sequencing and electron diffraction studies had shown that bacteriorhodopsin consisted of seven transmembrane-spanning α-helices (Ovchinnikov, 1982).

2.6.11 A G-protein-coupled receptor superfamily

The existence of a large family of GPRs was initially based on the structural and sequence homology of two GPRs, bovine rhodopsin and hamster βAR (Lefkowitz et al., 1986). It seemed reasonable to suppose that other adenylate cyclase-coupled receptors would also have the seven transmembrane-spanning α-helical structure. Moreover, the other members of the opsin family (Nathans et al., 1986), the putative yeast pheromone receptors (Nakayama et al., 1985) and the human *mas* oncogene (Young et al., 1986) all exhibited a seven TM-spanning domain structure. The GPR superfamily is growing daily as new members are constantly being identified and cloned and so far includes receptors for such diverse ligands as the biogenic amines, peptide hormones, the tachykinins, arachidonic acid derivatives, light and odourants (Table 2.4).

GPR Sub-family	**Ligand**
Light	Retinal
Odourants	Olfactory stimuli
Biogenic amines	Dopamine
	Noradrenaline/Adrenaline
	Histamine
	Serotonin
	Acetylcholine
	Octopamine (*Drosophila*)
Tachykinins	Substance P
	Substance K
	Neuromedin K
Glycoprotein hormones	Thyrotrophin
	Luteinising hormone
	Follicle-stimulating hormone
Hypothalamic peptides	Thyrotrophin-releasing hormone
	Gonadotrophin-releasing hormone
	Growth hormone-releasing hormone
Brain/gut peptides	Parathyroid hormone
	Angiotensin
	Arginine/vasopressin
	Vasoactive intestinal polypeptide
	Bombesin/gastrin-releasing hormone
	Neurotensin
	Bradykinin
Arachidonic acid derivatives	Thromboxane
	Prostaglandin
Miscellaneous	Thrombin
	Endothelins
	Platelet-activating factor
	Cannabinoids
	cAMP (*Dictyostelium*)
	Adenosine
	Mating factors (Yeast)
	C_{5A} anaphylatoxin
	Interleukin (IL-8)
	N-formyl peptide

Note: Several receptors have been cloned for which no ligands have as yet been identified, these receptors are known as orphan receptors and will add to this list once their ligands have been discovered.

Table 2.4

Ligands for GPR subfamilies (adapted from Probst et al., 1992, Lefkowitz, 1991).

2.6.12 Structural features of the GPR family

Alignment of the sequences for the GPRs (Strosberg, 1991; Probst et al., 1992) has indicated several features common to most members of the family, see Fig. 2.12 (Dixon et al., 1986; Lefkowitz et al., 1986; Baehr & Appleby, 1986; Strange, 1988; Lefkowtiz, 1991; Dohlman et al., 1991; Strosberg, 1991; Probst et al., 1992). The general structure includes seven hydrophobic transmembrane domains connected by three extracellular and three cytoplasmic hydrophilic loops, an extracellular amino (NH_2) terminus of varying length and a cytoplasmic carboxy (COOH) terminal tail, also of varying length. The gonadotrophin-releasing hormone (GnRH) receptors however, have no C terminal tail (Tsutsumi et al., 1992; Eidne et al., 1992; Kakar et al., 1992; Chi et al., 1993).

The greatest sequence homology between different receptors occurs in the membrane-spanning domains, whilst the widest sequence divergence occurs in the three extracellular and three intracellular connecting loops and the NH_2-terminus and COOH-terminal tails. A large third intracellular loop, is another common characteristic.

2.6.13 Common functional properties of GPRs

The binding of a ligand to its specific receptor results in a conformational change in receptor structure leading to the activation of a G-protein and subsequent stimulation or inhibition of signal transduction systems such as adenylate cyclase and/or phospholipase C. Another common feature of GPRs is their ability to adapt to long-term stimulation by the same ligand, known as desensitisation. Receptor phosphorylation appears to play a critical role in this process (Gilman, 1987; Strange, 1988; Strosberg, 1991; Probst et al., 1992).

2.6.14 Functional domains and chimaeric receptors

The initial reconstitution experiments performed with the individual components of the G-protein-coupled signal transduction system (Lefkowitz et al., 1983) were not sensitive enough for detailed analysis of the various molecular interactions involved between receptor, G-protein and catalytic enzyme.

Molecular biology has provided a powerful new tool for studying these interactions. The use of chimaeric receptors formed from regions of

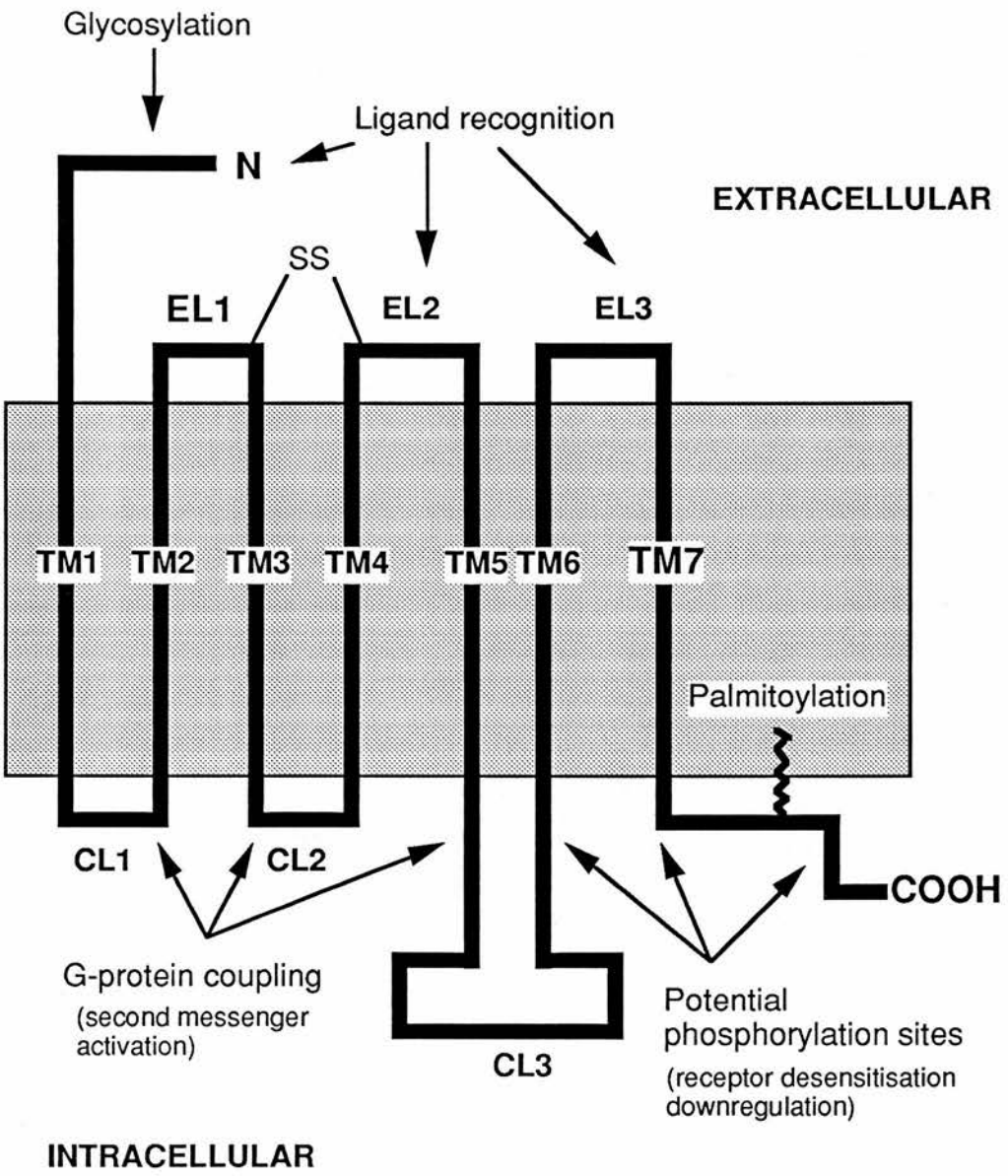


Fig. 2.12

Schematic representation of a G-protein-coupled receptor illustrating the proposed seven transmembrane (TM) topography with three extracellular loops (EL) and three intracellular loops (CL) and putative functional domains, determined by site-directed mutagenesis and chimaeric receptor analysis.

different receptor sub-types has enabled the identification of separate domains involved in ligand binding and G-protein coupling. Much of this work was initially performed with the adrenergic receptors (Ward et al., 1990). Combining different regions of the α_2AR, which couples to G_i and inhibits adenylate cyclase, with the β_2AR, which couples to G_s and stimulates adenylate cyclase, produced a receptor that combined features of the two original proteins. The binding of an α_2AR agonist to one construct stimulated adenylate cyclase, normally a βAR response (Kobilka et al., 1988). This experiment enabled tentative identification of a role for transmembrane 7 (TM7) in ligand binding and the NH_2-terminal region of TM5 to the COOH-terminal region of TM6 in G-protein coupling.

Chimaeric receptors formed from hamster β_1 and β_2ARs indicated that the region around TM4 and TM5 contributed to subtype-specific binding of receptor agonists, but could not identify any one amino acid responsible for agonist differentiation (Dixon et al., 1989).

Combining different domains of the tachykinin receptors, substance P and substance K, indicated that the domains responsible for ligand binding of any tachykinin covered TM2 to TM7, with a region extending from TM2 to the third extracellular loop (EL3) and a minor section of the extracellular NH_2-terminal domain selecting for a particular tachykinin (Yokota et al., 1992).

2.6.15 Deletion and site-directed mutagenesis

Deletion and site-directed mutagenesis studies are further examples of molecular techniques that have helped to determine more accurately, domains, and even specific amino acids, involved in ligand-binding and G-protein coupling (Ward et al., 1990). Deletion mutagenesis, involving the removal of small regions of a receptor, has indicated that the ligand-binding domain of the βAR does not directly involve the hydrophilic loops of the molecule as their absence has no effect on ligand-binding (Dixon et al., 1987). Site-directed mutagenesis has been widely used to pinpoint interactions between specific amino acid residues by replacing conserved residues with other amino acids of a different size or with a different functional side-group (Ward et al., 1990). This type of mutation can provide useful functional information, although the replacement amino acids must be selected carefully, as major conformational changes in the

receptor could mask the effects of the chosen residue. Again, much of the original work was performed with the adrenergic receptors, but this technique is now widely used.

The catecholamine group of ligands (DA, adrenaline and noradrenaline) are protonated amines and it was suggested that the ligand-binding site should contain an acidic residue to counteract the amine group. Therefore, substitution of aspartate (Asp) and glutamate (Glu) residues in the transmembrane domains with neutral amino acids helped to determine their importance. Only two substitutions had any effect, Asp^{79} to Ala in TM2 of the βAR resulted in a tenfold decrease in receptor agonist affinity, but had no effect on antagonist binding. Asp^{113} to Ala in TM3 resulted in a dramatic decrease in the affinity of the receptor for both agonists and antagonists. Response was measured by the decrease of adenylate cyclase stimulation (Strader et al., 1987a; Strader et al., 1988). The TM3 Asp has since been found to be a conserved residue in all catecholamine receptors, but not in receptors whose ligands are not biogenic amines (Tota et al., 1991; Probst et al., 1992).

Several conserved serine residues in TM5 of the catecholamine receptors have also been implicated in ligand binding by this approach (Strader et al., 1989a). Two conserved threonines in TM5 of the acetylcholine (ACh) muscarinic receptors were positioned in the predicted plane of ligand binding near the ligand's ester group, but were found to face away from the predicted binding site (Wess et al., 1991; Brann et al., 1993).

An asparagine in TM6 of the muscarinic receptors is thought to interact with the ester group of ACh and is not found in any other receptor class (Hibert et al., 1991). Recent receptor modelling by Brann et al. (1993) has demonstrated that this TM6 Asn is ideally located in the membrane pocket for interaction with the ester group of the ligand.

Site-directed mutagenesis has also supported the involvement of TM7 in ligand binding (retinal is covalently bound to a lysine in TM7 of rhodopsin). The aromatic amino acids phenylalanine (Phe^{290}) in TM6 and/or tyrosine (Tyr^{326}) in TM7 of the βAR may interact with the phenyl ring of serotoninergic and adrenergic ligands (Dixon et al., 1989; Strader et al., 1989b).

2.6.16 Ligand-binding and GPR subfamilies

The chromophore retinal lies in a hydrophilic pocket formed by the inner surfaces of the TM domains of rhodopsin, and when stimulated by a photon of light, produces a conformational change in the structure of the receptor (Thomas & Stryer, 1982; Findlay & Pappin, 1986). Dixon et al., (1986) proposed a similar model of a binding pocket formed by the hydrophobic TM domains encasing a hydrophilic core, for the interaction of the catecholamines with the βAR. This model was based on sequence homology of the postulated transmembrane domains 5, 6 and 7 (the retinal-binding site of rhodopsin) with the same region of the βAR and seems a likely model for receptors binding to small ligands such as the peptide hormones and neurotransmitters.

Within the GPR superfamily, several interesting subfamilies exist (Lefkowitz, 1991). The glycoprotein hormones, LH, FSH, and TSH belong to a family with unusually long extracellular NH_2-terminal domains (Probst et al., 1992), the TSH receptor NH_2-terminus is 398 amino acids long (Parmentier et al., 1989). These hormones are large, heterodimeric proteins unlikely to 'fit' into a transmembrane binding pocket. The extracellular NH_2-terminal domains are rich in cysteine residues that may form disulphide bonds to stabilise the tertiary structure of this putative ligand-recognition domain (Xie et al., 1990; Nagayama et al., 1991).

Another proposed sub-family, based on ligand-binding characteristics, is the tachykinin group (substance P, substance K and neuromedin receptors). These peptides are intermediate in size between the neurotransmitters and glycoproteins, and Yokota et al. (1992) have proposed an intermediate ligand-binding mechanism with the transmembrane domains TM2, TM3 and TM4 together with part of the extracellular NH_2-terminus involved in recognition and binding (Yokota et al., 1992).

A further variation on ligand-binding has been illustrated by the cloning of the thrombin receptor (Vu et al., 1991). This receptor was also found to have a large, extracellular NH_2-terminal domain, but contained an unusual extra feature. Thrombin, a platelet aggregation factor, was shown to cleave the NH_2-terminus of the receptor, creating a new NH_2-terminus that acted as a 'tethered' ligand and could activate the receptor.

2.6.17 Molecular modelling of the ligand binding domain

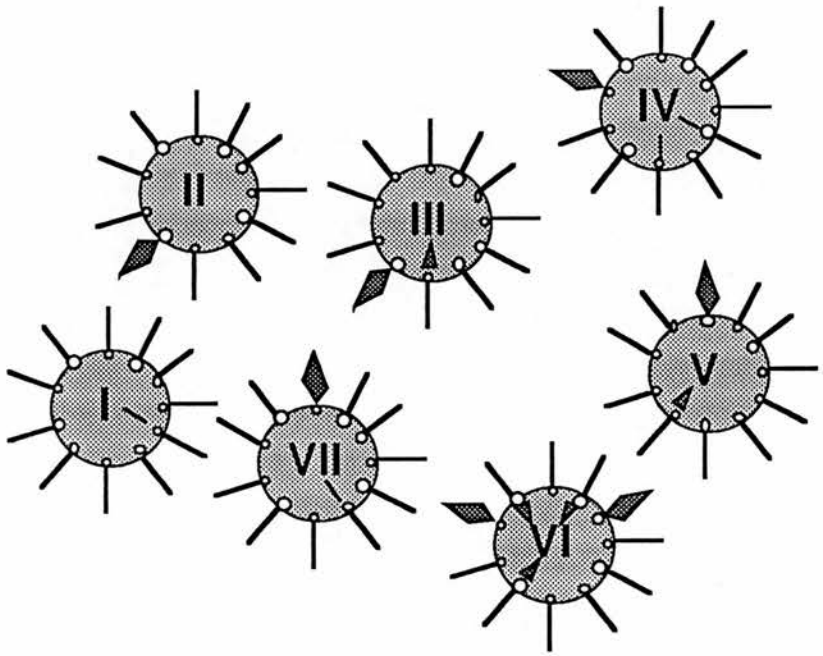
Bacteriorhodopsin is the only seven transmembrane domain protein that has been crystallised so far (Henderson et al., 1990) and three-dimensional models for the GPR ligand-binding pocket are generally based on its structure. Helical wheel models of receptors (Baldwin, 1993) see Fig. 2.13, can be used to study the binding pocket in which conserved residues can be arranged so that their side-chains face into the 'pocket' with most of the non-conserved residues facing into the lipid bilayer. Analysis of wheel models demonstrated that the seven transmembrane domains can be arranged to form a pocket with a hydrophilic centre into which the ligand may fit (Hulme et al., 1990). This information can be used for each receptor to further the study of ligand-receptor interactions. Recently, computer modelling has been developed to facilitate the work (Findlay & Eliopoulos, 1990; Brann et al., 1993).

Although site-directed mutagenesis studies have tried to define amino acids involved in ligand binding that are common to receptors of a particular group, it is becoming apparent that each ligand may define its own particular binding site depending on the precise interactions between each functional group of the ligand and the amino acid residues of the domains with which the ligand binds.

2.6.18 Cytoplasmic domains and G-protein coupling

The methods used to study the ligand binding domain have also been used to identify the determinants for G-protein coupling. Receptor-G-protein interactions are presumed to occur at the inner surface of the plasma membrane and accordingly the intracellular loops and/or COOH-terminal tail were predicted to be involved in G-protein coupling. Initial experiments involving proteolytic cleavage of the large third cytoplasmic loop (CL3) of rhodopsin abolished coupling with the G-protein transducin, supporting this assumption (Findlay & Pappin, 1986).

Deletion mutagenesis studies with the βAR extended the theory and showed that the important domains for G-protein coupling and adenylate cyclase activation were the regions of CL3 adjacent to the plasma membrane, see Fig. 2.12 (Dixon et al., 1987). The critical residues for activation of adenylate cyclase were eight amino acids forming the junction between TM5 and the NH_2-terminus of CL3 (Strader et al.,



Symbols used:

- Polar residue conserved in more than 10% of GPRs
- Residue conserved in more than 85% of GPRs
- O Size of circle indicates depth of residue within the membrane
- —— Bar indicates non-conserved residues
- — Internal bar indicates residue conserved in more than 65% of GPRs

Fig. 2.13

Helical wheel model showing the positions of the seven helices of a G-protein-coupled receptor according to the model of Baldwin (1993). The helices are generally shown as three layers, viewed from the inside of the cell. This diagram shows only one layer.

1987b) and twelve amino acids at the COOH-terminus of CL3 (Dixon et al., 1987).

The muscarinic m1 receptor is coupled to a G-protein that stimulates phospholipase C and the m2 receptor is linked to the adenylate cyclase system via an inhibitory G-protein. Chimaeric receptor experiments involving the interchange of CL3 and seven amino acids from the COOH-terminus of TM5 between the m1 and m2 muscarinic receptors, altered the coupling specificity without changing the pharmacology (Hulme et al., 1990). More recently, the NH_2-terminal seventeen amino acids of CL3 of the m3 muscarinic receptor have been defined as an important domain for coupling to the PI pathway (Brann et al., 1993). This region shows the greatest sequence divergence between the muscarinic receptors linked to the PI pathway (m1, m2 and m3 subtypes) and those linked to the adenylate cyclase pathway (m2 and m4) (Hulme et al., 1990).

Site-directed mutation of a conserved proline to a threonine in CL2 of the βAR did not affect agonist binding, but did cause a reduction in adenylate cyclase stimulation (O'Dowd et al., 1988) while mutation of the highly conserved Asp^{130} in the COOH-terminal region of CL2 of the βAR produced a receptor displaying a partial agonist phenotype that was uncoupled from its G-protein (Dixon et al., 1988; Fraser et al., 1988).

2.6.19 Transmembrane domains and G-protein coupling

The Asp^{79} in TM2 of the βAR, conserved in most GPRs appears to be necessary for G-protein activation as well as ligand binding. It is involved in the Na^+ and H^+ modulation of receptor coupling in the DA-Rs (Neve, 1991) and the α_2AR (Horstman et al., 1990). A conserved Cys residue in TM6 has also been implicated in βAR-activated signal transduction (Fraser, 1989).

2.6.20 Peptide competition studies and G-protein coupling

Synthetic peptides corresponding to sections of the hydrophilic loops have been used to map regions of the GPRs that may physically contact G-proteins. These peptides have helped to determine whether loss of function due to a particular receptor mutation has been due to loss of contact with a G-protein, or an alteration in the receptor's tertiary

structure. Peptides derived from the membrane-proximal regions of CL1, CL2 and CL3 and the COOH-terminal tail of receptors have implicated these regions in contact with G-proteins (Dalman & Neubig, 1991; Palm et al., 1990).

Mastoparan, an amphipathic helix-forming peptide derived from wasp venom is able to directly activate the G-protein G_o (Higashijima et al., 1990). The membrane-proximal regions of CL3 are also predicted to form amphipathic helices (Strader et al., 1987; Higashijima et al., 1990) and ligand binding may produce a conformational change in the receptor, exposing the amphipathic helices to the G-protein (Strader et al., 1989b). This may explain why receptors with little sequence identity, even in the suspected G-protein coupling domains, can activate the same G-protein (Segrest et al., 1990). Recent experiments with the dopamine D1, D2 and β_1ARs, however, has suggested that an amphipathic helical structure does not represent the main structural determinant for interaction with G-proteins (Voss et al., 1993).

To date, all of the intracellular, cytoplasmic domains as well as certain amino acids in the transmembrane domains have been linked to G-protein coupling and activation of signal transduction pathways, and so far there does not seem to be a specific domain conserved in all receptors for G-protein coupling. However, in spite of the evidence presented by Voss et al. (1993), it seems likely that secondary structure does play an important role in G-protein interactions.

2.6.21 Covalent modifications to GPRs

As well as the primary structural features of GPRs, post-translational covalent modifications at particular conserved amino acid residues may play an integral role in the processes of ligand binding and signal transduction. Such modifications include N-linked glycosylation, palmitoylation, disulphide bond formation and phosphorylation.

2.6.22 N-linked glycosylation

N-linked glycosylation involves the addition of carbohydrate residues to the amino side-chains of asparagine (Asn) residues and is known to be important in molecular and cellular interactions of plasma membrane

proteins. Potential glycosylation sites (Asn-X-Ser/Thr) are generally found in the extra-cellular NH_2-terminal domain of GPRs, and may be involved in the correct expression of the receptor within the membrane (Kornfeld & Kornfeld, 1985; Herron & Schimerlik, 1983). Tunicamicin, a glycosylation inhibitor, caused depletion of cell-surface muscarinic receptors and decreased the expression of the βAR, but had no effect on ligand binding (Dixon et al., 1987; Liles & Nathanson, 1986). The glycoprotein hormones have large extracellular NH_2-terminal domains, particularly rich in glycosylation sites (Parmentier et al., 1989; McFarland et al., 1989; Sprengel et al., 1990) which may be involved in ligand interactions or the correct expression of the receptor in the membrane (Liles & Nathanson, 1986).

2.6.23 Disulphide bond formation

A pair of cysteine residues in the extracellular loops EL1 and EL2, is a striking feature of most GPRs, with the exception of the human *mas* oncogene which binds angiotensin, and the orphan receptors, human endothelial cell GPR and human thoracic aorta GPR (Probst et al., 1992). There is evidence from experiments with disulphide reducing agents that these two residues form a disulphide bond and stabilise the correct conformation of receptors. At least two such bonds have been implicated in the normal ligand binding and cell surface expression of the β_2AR (Scattergood et al., 1987; Fraser, 1989; Dohlman et al., 1990).

2.6.24 Palmitoylation

Another conserved feature of many GPRs is the presence of several conserved Cys residues in the COOH-terminal tail. These residues are generally located in the membrane-proximal regions of the cytoplasmic tail, and in both the βAR and rhodopsin, are covalently modified by palmitoylation (Mouillac et al., 1992; Ovchinnikov et al., 1988). The addition of palmitate, a fatty acid, to newly synthesised proteins occurs after exit from the endoplasmic reticulum and it has been suggested that membrane-binding of a palmitic acid residue could create an extra loop in the COOH-terminal tail, which may anchor the receptor in the membrane (Findlay & Eliopoulos, 1990; O'Dowd et al., 1989) see Fig. 2.12.

2.6.25 Phosphorylation and receptor desensitisation

After prolonged exposure to a particular stimulus, signal transduction pathways often exhibit a reduced response. This ability to adapt or desensitise may occur at the level of the receptor and the observed desensitisation of systems to certain drugs is a limiting factor in the use of GPR agonists as therapeutic agents.

Receptor desensitisation involves at least three agonist-induced alterations in the properties of a receptor (Sibley & Lefkowitz, 1985; Strader et al., 1989b; Dohlman et al., 1991). Prolonged exposure to agonist results in down-regulation of receptors, and reflects the internalisation of receptors away from the plasma membrane. This is a slow process occuring over a number of hours and does not account for the rapid loss of intracellular response. The agonist also triggers a faster (within minutes) sequestration of receptor into a cellular compartment distinct from the plasma membrane from where it is quickly recycled to its active form. Finally, the agonist also induces a rapid modification of the receptor such that it functionally uncouples from the G-protein. This uncoupling may be mediated by receptor phosphorylation (Strader et al., 1987b; Bouvier et al., 1988; Hausdorff et al., 1989; Dohlman et al., 1991).

2.6.26 Long-term desensitisation

Down-regulation of receptor numbers on the cell surface occurs over a number of hours. Receptors do not have to be phosphorylated or sequestered to be down-regulated (Hausdorff et al., 1991). Both cAMP-dependent and independent mechanisms have been implicated in receptor down-regulation and may include alterations in mRNA stability as well as in regulating rates of gene transcription, protein synthesis and receptor degradation (Chuang, 1984; Hadcock & Malbon, 1988; Collins et al., 1989). Mutations of the human β_2AR that interfered with G-protein coupling also interfered with receptor down-regulation but not sequestration (Campbell et al., 1991).

2.6.27 Sequestration of receptors

Sequestration is another process involved in the negative regulation of receptors, occuring within minutes of exposure to agonist stimulation. It may involve the removal of the receptor from the cell-surface to an

unknown site within the cell (Wang et al., 1989). Upon removal of agonist stimulation, sequestered receptors are rapidly recycled to the cell surface in a fully functional state (Hertel et al., 1983).

Ligand-bound TRH-Rs appear to be sequestered while still coupled to the G-protein-phospholipase C complex (Nussenzveig et al., 1993a), with two domains in the COOH tail affecting this process (Nussenzveig et al., 1993b). This is in contrast to adenylate cyclase-coupled receptors which are believed to uncouple from the G-protein before internalisation (Birnbaumer et al., 1990). Receptor sequestration can be blocked without affecting desensitisation (Hertel et al., 1985) thus there appears to be no correlation between phosphorylation and sequestration (Strader et al., 1987c).

2.6.28 Short-term desensitisation

Exposure of cells expressing the βAR to low concentrations of agonist (nM) reduces the sensitivity of cells to the agonist without changing the maximal responsiveness. Low concentrations of agonist appear to activate only an agonist-independent kinase such as the cAMP-dependent protein kinase A (PKA) (Hausdorff et al., 1989; Clark et al., 1989). PKA has been shown to phosphorylate the βAR at ArgArgSerSer residues in CL3 and the membrane-proximal region of the COOH tail (Hausdorff et al., 1989). Site-directed mutagenesis of these sites resulted in reduced receptor phosphorylation and impaired desensitisation, but did not affect coupling of the β_2AR to G_s (Hausdorff et al., 1991; Clark et al., 1989). Protein kinase C (PKC), coupled to the PI/Ca^{2+}-calmodulin pathway also acts in an agonist-independent manner to phosphorylate receptors (Richardson & Hosey, 1990; Bouvier et al., 1991).

Exposure of cells expressing the βAR to high concentrations of agonist (μM) decreases both the sensitivity and maximal responsiveness and activates an agonist-dependent kinase known as βARK (Benovic et al., 1986) which phosphorylates the βAR at Ser/Thr residues at the distal end of the COOH tail (Bouvier et al., 1988; Benovic et al., 1989). βARK phosphorylation is thought to facilitate the binding of the cytosolic protein β-arrestin (Lohse et al., 1990) to the receptor, which seems to further disrupt G-protein coupling. βARK can also phosphorylate receptors other than the βAR, and may be ubiquitous (Richardson et al., 1993; Kwatra et

al. 1993). Thus there appears to be two independent pathways involved in desensitisation of receptors.

2.6.29 Homologous and heterologous desensitisation

Short-term desensitisation can be further separated into homologous and heterologous responses (Sibley & Lefkowitz, 1985; Dohlman et al., 1991). Homologous desensitisation describes the reduced responsiveness to the original stimulus, while heterologous desensitisation involves the incubation of receptor with one agonist attenuating the response to other agonists operating through distinct receptors. Heterologous desensitisation of the βAR appears to involve the cAMP-dependent PKA and follows any stimulus that raises cAMP levels in the cell (Dohlman et al., 1991).

2.6.30 GPRs and disease

GPRs mediate a wide variety of physiological actions (Table 2.4) including hormonal stimulation and neurotransmission, olfaction and vision via ubiquitous intracellular signalling pathways. It is possible, therefore, that defects in these receptors might be responsible for certain pathologies. Recently two reports have described spontaneous mutations that result in the constitutive activation of GPRs and human disease. Permanantly activated TSH-Rs result from somatic mutations in the membrane-proximal region of the third cytoplasmic loop, and are found only in tissue from hyperfunctioning thyroid adenomas (Parma et al., 1993). Male precocious puberty is due to a mutation in TM5, which constitutively activates the LH-R resulting in Leydig cell hyperplasia and production of testosterone in the testis (Shenker et al., 1993). A mutation in TM7 which prevents binding of retinal to rhodopsin leads to constitutive activation of the receptor and retinal degeneration (Robinson et al., 1992). In mice, point mutations in CL1 and TM2 result in constitutive activation of the melanocyte-stimulating hormone receptor leading to hyper-pigmentation (Robbins et al., 1993).

Interestingly, the mutations in the TSH-R occur in the same positions as mutations produced in the α_{1B}-AR *in vitro* (Allen et al., 1991) which induced focus formation in transformed fibroblasts without agonist stimulation of the receptor. Similar mutations in the β_2-AR (Samama et

al., 1993) and the α_2-AR (Ren et al., 1993) also resulted in constitutive receptor activation. It appears, therefore, that certain amino acid residues must be responsible for preventing receptors from coupling to G-proteins until stimulated by an agonist. When these residues are mutated, the receptor conformation somehow changes to a permanantly activated state (Lefkowitz, 1993a,b).

Over-expression of wild-type serotonin (a neurotransmitter) receptors in non-neural cell lines results in transformation when stimulated by agonist (Julius et al., 1989) suggesting that ectopic expression of receptors might cause them to act as oncogenes. One example of a potential transforming GPR is the human *mas* oncogene which binds angiotensin, a peptide responsible for regulating blood pressure (Young et al., 1986; Jackson et al., 1988). Recently three GPR homologues have been identified within the coding region of the human cytomegalovirus (HCMV) (Chee et al., 1990). HCMV is a herpes virus, but can cause congenital and neonatal disease, and is a dangerous pathogen in individuals with a deficient immune system.

The study of GPR-activating mutations as well as wild-type receptors that act as oncogenes will be useful in determining the causes of, and possible therapies for, receptor-related diseases. These mutations will also be helpful in elucidating the structural basis for agonist-stimulated receptor activation in wild-type receptors.

2.6.31 Evolution of GPRs

Comparison of GPR sequences with those of bacteriorhodopsin, which has seven transmembrane domains, but is not G-protein-coupled, has revealed fairly low sequence homology. This is not surprising, as GPRs must have diverged from bacteriorhodopsin approximately 1.5 billion years ago when prokaryotes and eukaryotes diverged. The observation by Pardo et al., (1992) that TM7 of bacteriorhodpsin is most homologous to TM3 of GPRs led them to propose that exon shuffling occured during the evolution of the GPRs with the result that the TM helices would require a different folding pattern. Taylor & Agarwal (1993) suggested that the GPRs may instead have evolved by a gene duplication event from bacteriorhodopsin such that TM5 to TM7 arose as duplicates of TM1 to TM3, which also explains why TM3 of the GPRs is similar to TM7 of bacteriorhodopsin. Since most of

the three-dimensional work on GPRs has been based on the structure of bacteriorhodopsin, this question needs to be resolved.

Comparison of GPR genes that contain introns has shown that introns are often in similar positions, even in genes of different GPR families, and generally occur outside the transmembrane domains (Probst et al., 1992, Strosberg, 1991) supporting the idea that members of the GPR family may have evolved from a common ancestor. The yeast pheromone receptors are also seven transmembrane domain GPRs with structural and functional, if not sequence, similarity to the rest of the GPR superfamily (Blumer et al., 1988), implying an ancient origin for the GPR genes.

2.6.32 Multiple subtypes for GPRs

The first GPR genes to be cloned, the βAR and muscarinic receptors, were intronless (Dixon et al., 1986; Hulme et al., 1990), however introns have been described in GPR genes such as the dopamine D2, D3 and D4 subtypes (Civelli et al., 1993), the tachykinins (Gerard et al., 1990; Gerard et al., 1991), the thyrotrophin-releasing hormone receptor (Duthie et al.,1993b), the opsins (Nathan & Hogness, 1984, Nathans et al., 1986), the prostaglandin PGE_2 EP3 receptor (Namba et al., 1993) and the pituitary adenylyl cyclase-activating polypeptide (PACAP) type I receptor (Spengler et al., 1993).

In addition to multiple receptor subtypes for a particular ligand, GPR genes containing introns are capable of even greater structural and functional diversity by a process known as alternative splicing. The first example of an alternatively spliced GPR was the dopamine D2 receptor which has a 29 amino acid insert within the third cytoplasmic loop (see Chpt. 7 for further discussion). Another instance of a functional role for alternative splicing has been clearly demonstrated for the PACAP receptor (Spengler et al., 1993). Five splice variants of this receptor have been identified with insertions at the COOH-terminal end of CL3, a region implicated in G-protein coupling. These inserts are created by the presence, or absence, of one or two, 84 nucleotide casettes termed 'hip' and 'hop'. Expression studies showed that these splice variants have individual patterns of adenylate cyclase and phospholipase C stimulation.

Alternative splicing of the prostaglandin PGE_2 EP3 receptor produces different COOH-terminal tails resulting in differential G-protein coupling (Namba et al., 1993) and it is possible that the various forms of the TRH-R (Straub et al., 1990, Zhao et al., 1992, de la Peña et al., 1992a,b; Sellar et al., 1993; Duthie et al., 1993a,b; Matre et al., 1993, Yamda et al., 1993) may be able to couple to different G-proteins or be differentially regulated due to the variation in the length of their COOH tails. The genomic structure of the TRH-R will be discussed in more detail in Chpt. 6.

2.7 Summary

The molecular cloning of increasing numbers of GPR cDNAs and their genes has greatly accelerated the analysis of GPR ligand-binding, G-protein-coupling and regulatory mechanisms. Site-directed mutagenesis and the use of chimaeric receptors have helped to determine domains involved in specific functional events, including ligand binding, G-protein-coupling and receptor phosphorylation. Three-dimensional modelling techniques have aided the study of receptor-ligand binding domains, whilst the expression of mutant receptors in mammalian cell lines has furthered the study of receptor activation of intracellular signal transduction systems.

3 General Materials and Methods

The techniques described in this chapter are general methods used for the isolation and characterisation of cloned DNA sequences from commercially prepared libraries. Specific information is given in the relevant chapters. A detailed list of buffers is included in Appendix I and a list of chemical and equipment suppliers in Appendix II.

3.1 Introduction

To determine the sequence of a DNA molecule, it must first be cloned in order to make millions of copies of one molecule. DNA from the required source (genomic DNA or complementary DNA (cDNA), a stretch of DNA which faithfully copies a stretch of RNA) is ligated into an appropriate vector, such as a plasmid or bacteriophage. The vector and DNA is known as recombinant since the foreign DNA is cut with enzymes called restriction endonucleases, and recombined into a compatible site in the vector. A single molecule of recombinant DNA is then introduced into a host bacterium. Plasmids are transformed into bacteria by mechanically permeabilising the host cell walls, while bacteriophage have the ability to infect their hosts. An *E.coli* bacterium will divide approximately every twenty minutes, and as each bacterium may contain several hundred copies of the recombinant molecule, millions of identical recombinant molecules can be produced in a short time.

Over the last twenty years, progressively simpler, but more powerful, techniques for cloning genes have been developed. Improved host bacterial strains and vectors have been produced, together with a greater understanding of mammalian cell culture, synthetic oligonucleotide synthesis and enzymes that operate on DNA and RNA. The recent development of the polymerase chain reaction (PCR) in the mid-1980's (Saiki et al., 1985, Mullis et al., 1986, Mullis and Faloona, 1987) with its ability to detect very rare DNA sequences and amplify them without resorting to cloning, has brought about a technical revolution in molecular biology.

Once the desired gene has been cloned, determination of its nucleotide sequence enables the primary structure of the resultant protein

or polypeptide to be elucidated. The amino acid sequence can then be used to predict the conformational structure and the functional domains of the molecule. For a G-protein-coupled receptor, embedded in the cell membrane, the distribution of the hydrophobic and hydrophilic residues allows its arrangement in the lipid bilayer to be predicted (Kyte & Doolittle, 1982).

3.2 Library Screening - general principles

Homology screening of both genomic and cDNA libraries was performed using similar methods based on those described by Benton & Davis, 1977.

3.2.1 Preparation of host plating cells

A master plate was prepared by streaking 5µl of the appropriate *E.coli* host strain (in 50% glycerol, Clontech) on to a magnesium-free LB agar plate containing the required media additives, and incubating overnight at 37°C. A single, isolated colony was streaked on to a second LB agar plate and incubated overnight. A 10ml overnight culture was set up by innoculating 10ml of LB broth with 0.2% w/v maltose, 10mM $MgSO_4$ (Appendix I) with a single colony of *E.coli* and incubating with shaking overnight at 37°C. A control culture excluding *E.coli* was also set up to ensure that growth was due to the host bacteria and not contamination. Cells were spun down for 10min. at 2000rpm, the media decanted off, and the cell pellet carefully resuspended in 4ml of 10mM $MgSO_4$..

3.2.2 Screening Procedure

A total of $6x10^5$ plaques were screened for each library on two $23cm^2$ culture plates (Nunc). Host plating cells (400µl) were inoculated with $3x10^5$ pfu of the phage library, for each primary plate, and incubated at 37°C for 20min. to allow the phage to adsorb to the host cells. Molten LB top agar (50ml) was added to the inoculant, mixed well and spread evenly on to a $23cm^2$ culture plate containing LB bottom agar. The plates were incubated at 37°C overnight until plaques were visible.

The culture plates were refrigerated for an hour at 4°C to prevent the top agar from sticking to the nylon filters. Replica plaque lifts, with Biodyne A nylon filters (Pall, UK) were performed according to the

manufacturer's instructions. Each plate was overlaid with a nylon filter cut to size and the plaques allowed to adsorb to the filter for 1min. The filter was orientated using needle marks pricked through the filter on to the agar. It was then carefully removed and placed, plaque side up, on Whatman 3MM filter paper, saturated with high salt denaturing solution (Appendix I), for 5min., and on filter paper saturated with neutralising solution (Appendix I), for 5min. and left to air-dry on dry Whatman filter paper. A second filter was laid on to the culture plate for 3min., orientated with the same needle marks and treated in the same way as the first filter. A diagram of the method is shown in Fig. 3.1. The DNA was fixed to the filter by baking at 80°C for 30min. and/or cross-linking using the optimal cross-link setting on a Spectrolinker XL-1000 u/v crosslinker. Before cross-linking, the filters were wrapped in cling-film to protect the surface of the membrane. The primary culture plates were stored at 4°C for use after screening.

3.2.3 Prehybridisation

There are many methods available to hybridise radioactive probes to nucleic acids immobilised on solid supports such as nylon filters. The method used here was based on guidelines set by Sambrook et al. (1989). The dried, cross-linked filters were incubated in a small volume (just enough to cover the filter) of 15% formamide cDNA hybridisation buffer (see Appendix I) in a heat-sealed plastic bag at 65°C for a minimum of 2 hours.

3.2.4 Hybridisation

A radio-labelled, double-stranded rat cDNA probe (prepared as described in section 3.5) was denatured by boiling for 10min. and then placed immediately on ice. Following prehybridisation, the hybridisation buffer was decanted and replaced with buffer containing approximately $1x10^6$ counts/ml of the denatured probe. The filters were incubated overnight at 65°C in heat-sealed bags.

3.2.5 Washes

After hybridisation, the probe-containing buffer was carefully discarded and the filters washed firstly at low stringency (3XSSC, 0.1% w/v SDS) at

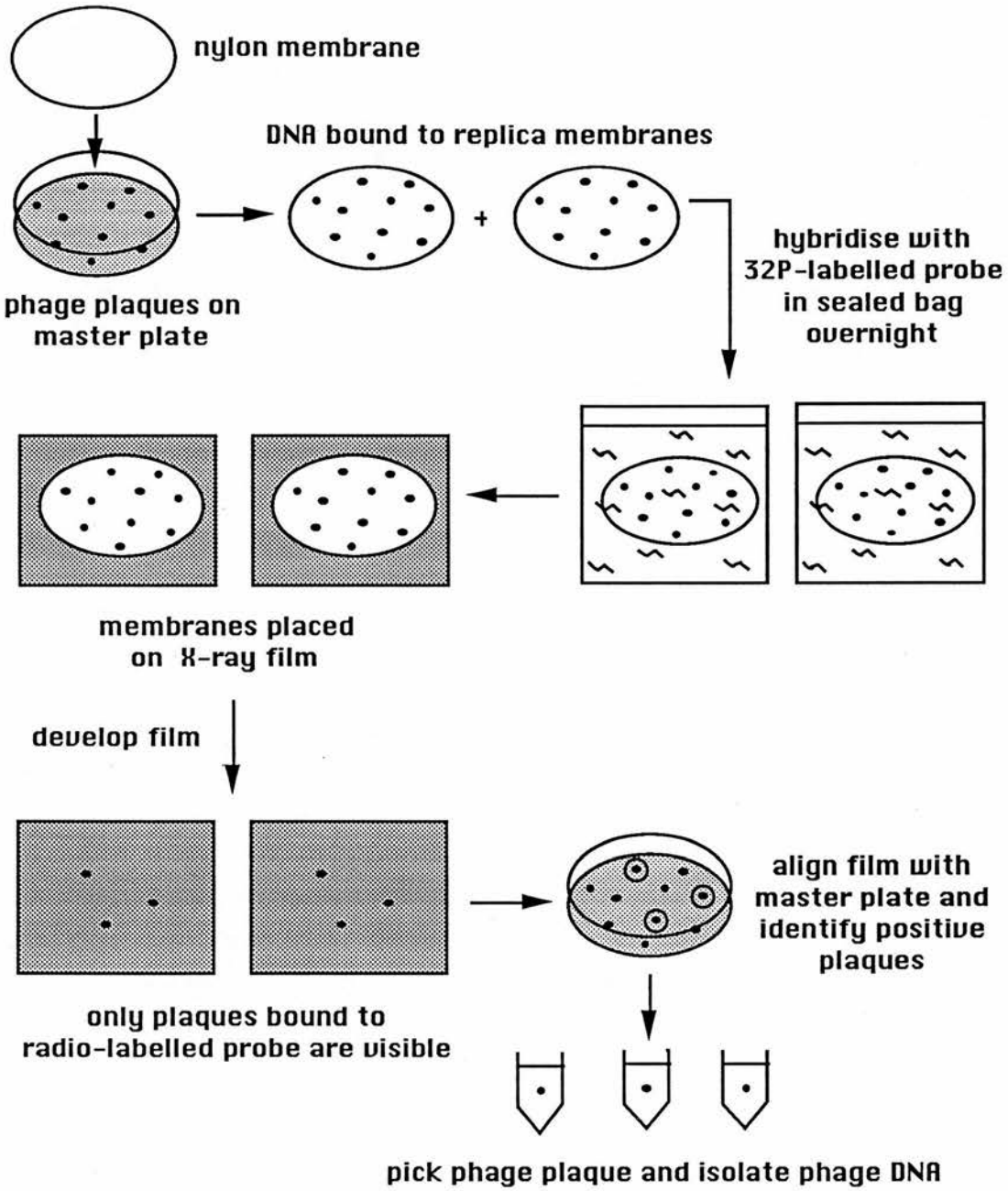


Fig. 3.1

Library screening. Several thousand phage plaques are overlaid with duplicate nylon membranes. Phage adhere to the membranes creating a precise replica of the plaque pattern. Membranes are incubated with a radio-labelled probe which binds to complementary sequences in the phage plaques. Membranes are washed to reduce non-specific binding. Positive clones appear as dark spots when membranes are exposed to x-ray film. Film is aligned with the master plate and positive plaques isolated.

room temperature for a total of 30min., changing wash buffer frequently and monitoring the level of radioactivity. The filters were then washed at higher stringency (1XSSC, 0.1% w/v SDS) at 65°C for 1 to 5min.

Once the background radiation had been reduced to between 5 and 10 counts per second, the filters were blotted quickly on Whatman 3MM paper, and making sure they were still damp, placed in heat-sealed plastic bags as filters cannot be reprobed once they have dried.

3.2.6 Autoradiography

The plastic-enclosed filters were placed in cassettes with two intensifying screens, overlaid with two X-OMAT™ AR scientific imaging films (Kodak) and exposed overnight at -70°C. The top film was developed with LX 24 X-ray developer (Kodak), fixed with FX-40 X-ray liquid fixer (Kodak) according to manufacturer's instructions and left to dry. If positive signals were visible, the second film was also developed, if not, it was left for a longer exposure time at -70°C.

3.2.7 Secondary screening

Films from replica filters were aligned using the needle orientation marks. Putative clones were identified as dark spots appearing in the same position on both films. Film was matched to the primary culture plate and positive areas marked. A square of agar was removed from the appropriate area and placed in 3ml SM buffer (Appendix I) at 4°C overnight to allow the phage to diffuse out of the agar into the buffer.

The phage-containing SM buffer was diluted, and titered on 150mm LB agar plates so that each plate contained approximately 500 plaques. Following overnight incubation, replica lifts, hybridisation and autoradiography was performed for each secondary plate as described previously. Individual positive plaques were identified and placed in 500µl of SM buffer with a drop of chloroform, at 4°C overnight.

3.3 Isolation and purification of bacteriophage DNA

3.3.1 Plate lysate preparation (small scale)

Approximately 10^5 pfu (1/20 of a resuspended plaque) was mixed with 0.1ml plating cells and incubated at 37°C. After 20min., 15ml molten LB

top agar was added, and the inoculum poured on to 150mm LB bottom agar plates. Plates were incubated overnight at 37°C. SM buffer (8ml) was added and the plates stored at 4°C for several hours with intermittent shaking. The SM buffer was harvested with a pasteur pipette and placed in a 50ml polypropylene tube (Falcon). Fresh SM buffer (2ml) was added to the plate, which was stored in a tilted position for 15min. The remaining SM buffer was harvested, combined with the first harvest and the plate discarded. To lyse any remaining intact plating cells, chloroform was added to 5% total volume to the pooled SM buffer, vortexed and centrifuged at 4000rpm for 10min. at 4°C. The supernatant was recovered, a drop of chloroform added, and stored as a stock lysate at 4°C.

3.3.2 Phage stock preparation

TM buffer (5ml) and DNAse 1 (150U) was added to 5ml of the stock lysate, mixed by gentle inversion and incubated for 15min. at room temperature to digest any bacterial DNA. To promote dissociation of bacteriophage particles from host cell debris, the lysate was incubated on ice with 1ml of 5M NaCl and 1.1g of PEG 6000 (ensuring that the PEG was completely dissolved) for 15min. The bacteriophage suspension was centrifuged at 8000rpm (in a Beckman centrifuge, Model J2-21, fixed angle rotor JA-20.1) for 15min at 4°C. The supernatant was poured off, the precipitated bacteriophage pellet resuspended in 150µl of TM buffer and transferred to an eppendorf tube. The PEG and cell debris was extracted from the bacteriophage suspension by vortexing with 150µl chloroform. After centrifuging for 5min. the upper aqueous layer was removed to a new tube and the chloroform extraction repeated.

NaCl was added to a final concentration of 0.5M together with 25mM EDTA, pH 8.0 and 175µl of stock-solution phenol (Appendix I) to remove the protein coat from the bacteriophage. This was vortexed and centrifuged for 5min. The upper aqueous layer was removed to a new tube and 175µl of chloroform added. After vortexing well, and centrifuging for 5min. the upper phase was again removed to a new tube. To precipitate the DNA, 0.1 volume sodium acetate, pH 5.2 and 2.5 volumes of absolute ethanol was added, mixed by gentle inversion and the tube placed on wet ice for 10min. The DNA was centrifuged at 4°C for 10min. at 14000rpm. The supernatant was carefully decanted off the

DNA pellet, and discarded. The pellet was washed with 75µl of 70% ethanol and centrifuged again at 4°C for 10min. The supernatant was discarded, the pellet dried under vacuum and resuspended in an appropriate volume (50µl) of TE buffer. The DNA was stored at -20°C prior to restriction digestion or polymerase chain reaction analysis (PCR).

3.3.3 Large scale liquid lysate preparation

This method was based on a procedure described by Malik et al. (1990) utilising double nuclease digestion and PEG precipitation.

Host plating cells (10ml, prepared as described previously) were inoculated with approximately $5x10^7$ pfu of phage for every 10^{10} plating cells and incubated at 37°C for 20min, shaking occasionally. A sterile growth flask containing 500ml of LB broth and 10mM $MgSO_4$ was inoculated with the infected plating cells, plugged with cotton wool and shaken overnight at 37°C.

Once the supernatant cleared, indicating lysis of the culture, 10ml of chloroform was added and the culture shaken for an additional 10min. at 37°C. The culture was cooled to room temperature, DNAse I and RNAse A each added to a final concentration of 1µg/ml and left to stand for 30min. These enzymes digested nucleic acids liberated from the lysed bacteria, which would otherwise trap phage particles.

NaCl (1M final concentration) was added to promote dissociation of phage from bacterial debris and for efficient precipitation of phage particles from PEG. The culture was left to stand for 1 hour at 4°C. Bacterial debris was removed by centrifugation at 8000rpm for 10min. at 4°C (in a Beckman JA-10 fixed angle rotor) in 250ml polypropylene bottles.

Final concentrations of 10% (w/v) PEG 8000 and 10mM $MgSO_4$ were added to the aqueous supernatant. The PEG was dissolved slowly at room temperature and then left to precipitate for 1 hour at 4°C. Phage was collected by centrifugation at 8000rpm for 20min., at 4°C, the supernatant discarded, and the pellet drained carefully of excess liquid. The pellet was resuspended in 7.5ml TM buffer for each 250ml bottle and transferred to a 50ml polypropylene tube (Falcon).

The PEG was extracted from the phage suspension by adding equal volumes of chloroform, vortexing well and centrifuging for 15min. at

3100rpm, 4°C in a swing out rotor (Omnispin R centrifuge, Sorvall Instruments). The upper aqueous phase containing the phage particles was collected, being careful not to disturb the PEG interface, and the chloroform extraction repeated.

Second nuclease digestion: DNAse I and RNAse A were each added to a final concentration of 0.1mg/ml and the phage suspension incubated at 37°C for 30min. Following digestion, the phage particles were lysed to release the DNA by incubating with 0.2% SDS and 10mg/ml proteinase K for 1 hour at 37°C.

The phage DNA was phenol/chloroform extracted and ethanol precipitated as described for the small scale preparation. DNA could also be further purified from proteins, salts etc. using the Promega Magic™ DNA Clean-Up System kit according to manufacturer's instructions.

3.4 Subcloning DNA into a plasmid vector

The TA Cloning™ System (Invitrogen) utilises the single deoxyadenosines added to the 3' ends of all PCR products by Taq polymerase, which allow a PCR product to be inserted into a specially designed plasmid vector, pCR™II, containing single 3' T-overhangs at the insertion site (Fig. 3.2).

A 1:1 and 1:3 molar ratio of vector to PCR insert was used in the ligation reactions which were performed according to manufacturer's instructions. The TA Cloning™ One Shot Cells (INVαF' cells) provided were transformed with 1µl of the ligation mix and transformants were selected on LB agar medium containing 50µg/ml ampicillin (Amp) and 50µl X-Gal (20µg/ml).

Insertion of the PCR product into the vector disrupts the *Ecoli* lacZ gene resulting in the appearance of white colonies. Blue colonies are the result of self-ligation of the vector and normal functioning of the lacZ gene which produces the enzyme β-galactosidase in the presence of an inducer such as the chromogenic substrate X-Gal (Sambrook et al, 1989).

Positive white colonies were picked and restreaked onto LB agar plates containing Amp and X-Gal, incubated overnight at 37°C and stored at 4°C as stock plates. The colonies were also subjected to plasmid lysis PCR as described in the next section.

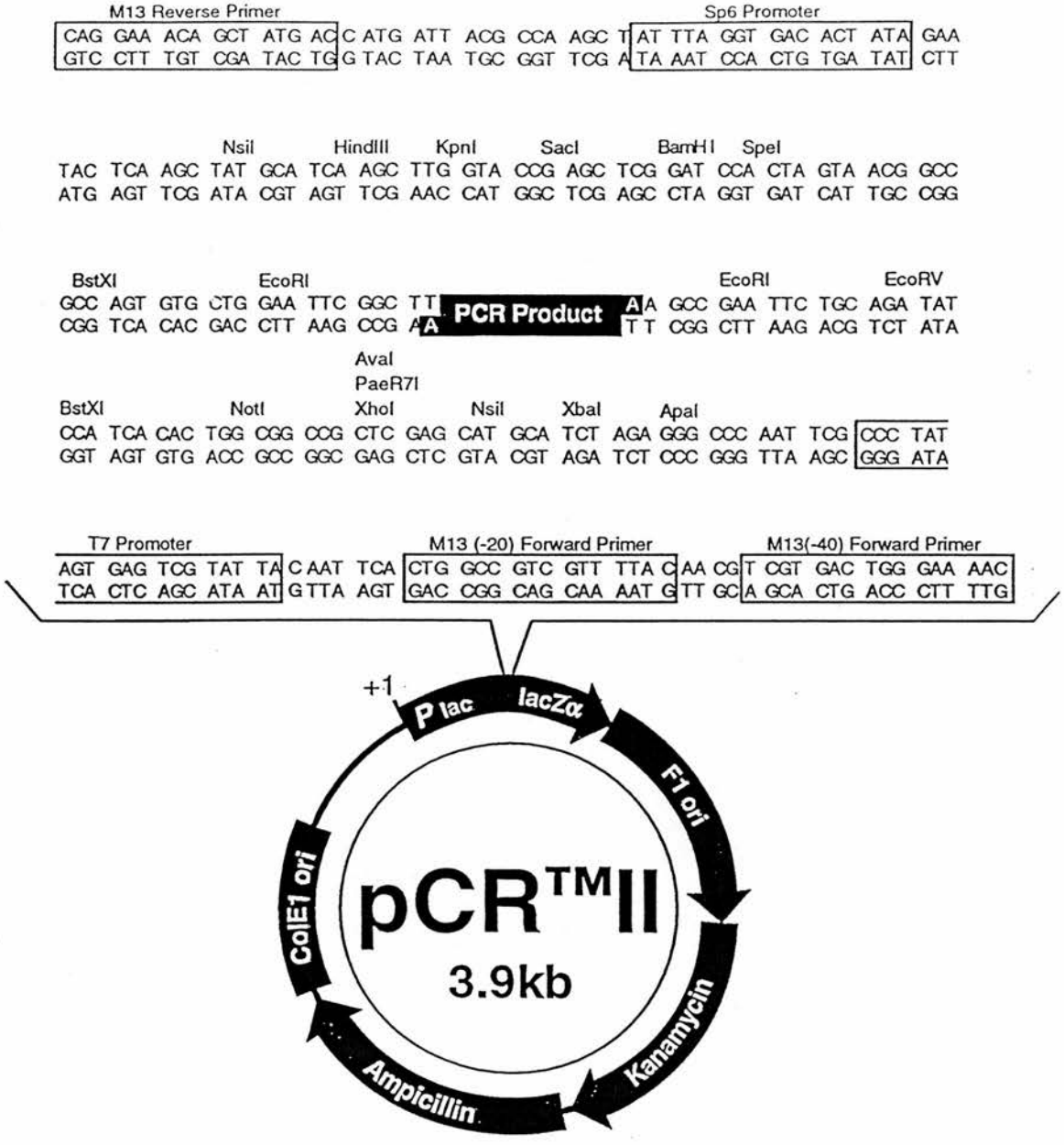


Fig. 3.2

TA cloning vector pCR™II (Invitrogen) used in subcloning polymerase chain reaction (PCR) products amplified from genomic or cDNA clones.

3.5 Plasmid lysis PCR

Positive colonies (see above) were picked from the agar plate with a sterile cocktail stick. Each colony was inoculated into 50µl of plasmid lysis buffer (Appendix I) in a 0.5ml Eppendorf tube. The lysis buffer/colony was heated to 95°C for 5min. and spun for 30sec. to pellet cell debris. The supernatant (5µl) was used as the template in a PCR reaction (see section 3.9). Appropriate oligonucleotide vector primers (SP6 and T7) were used to amplify subcloned DNA. This method was used to screen large numbers of colonies for the presence and size of the DNA insert without having to grow up and purify large amounts of plasmid DNA for restriction digestion analysis.

3.6 Purification of plasmid DNA

A culture of *E.coli* was prepared by inoculating 10ml LB broth (supplemented with appropriate antibiotics) with a single bacterial colony, infected with the required plasmid (section 3.4). The culture was incubated at 37°C overnight with shaking. DNA was purified with the Promega Magic Mini-Preps™ DNA Purification System according to manufacturer's instructions. This method involved the resuspension, lysis, and neutralisation of 3ml aliquots of the culture and produced approximately 10µg of plasmid DNA starting from a 1 to 3ml culture of *E.coli*. For larger amounts of DNA, a 10ml starter culture of *E.coli* was prepared and used to inoculate 500ml LB broth (supplemented with appropriate antibiotics). The Promega Magic Maxi-Prep™ kit yielded approximately 600µg of DNA from a 500ml starting culture of *E.coli*.

3.7 Analysis of purified DNA

3.7.1 Restriction enzyme digestion

In order to determine the size of isolated positive clones, the insert was released from the lambda vector by digestion with the appropriate restriction enzyme (see relevant chapters for details). Restriction enzymes were generally obtained from Boehringer Mannheim or Pharmacia (Appendix II). Restriction digests of purified DNA were carried out in 1X

restriction enzyme buffer in a total volume of 20µl and incubated for a minimum of 1 hour at 37°C.

3.7.2 RNAse treatment

To remove contaminating RNA, the purified, digested DNA was treated with DNAse-free RNAse A at a final concentration of 100µg/ml and incubated at 37°C for 30min. The digested DNA fragments were then separated on an agarose gel.

3.7.3 Agarose gel electrophoresis

An agarose gel of the appropriate percentage was chosen according to the expected size of DNA fragments to be separated (Table 3.1). For example a 0.7% w/v agarose gel (SeaKem) in 1XTBE, containing 0.3µg/ml EtBr was run at a constant voltage of 50V for several hours in 1XTBE electorphoresis buffer, to ensure good resolution of restriction fragments.

Amount of agarose in gel (%[w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5.0-60
0.6	1.0-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Table 3.1

Range of separation in gels containing different amounts of agarose (Sambrook et al., 1989).

Electrophoresis loading buffer was added to 10µl of each sample in order to estimate the position of the DNA in the gel. DNA markers (lambda - Hind III/Eco R1, and pGEM® - Promega) were also included to ascertain the size of digested DNA fragments. Following electrophoresis the

separated DNA fragments were viewed under ultraviolet light using a u/v transilluminator (Vilber Lourmat).

3.7.4 Southern blotting (alkali blotting procedure)

Apparatus was set up as depicted in Fig. 3.3. Two pieces of Whatman 3MM filter paper were cut and soaked in alkali transfer buffer (0.4M NaOH). Each wick was laid over the glass plate and bubbles rolled out with a glass rod. The gel was placed on top of the wicks and bubbles rolled out as before. (Gels containing DNA fragments of greater than 10kb were first placed in 1M HCl until the loading buffer dyes turned yellow, left for an additional 10min., and rinsed in distilled water). Cut autoradiograph film was inserted between the gel and the wicks to ensure that the buffer soaked up through the gel. Hybond N^+ nylon membrane (Amersham), cut to size, was placed on top of the gel and bubbles removed. Four pieces of Whatman 3MM paper were placed on top of the Hybond membrane, the first sheet having been soaked in transfer buffer. Paper towels were laid on top of the Whatman paper and a heavy weight placed on top. The blot was left overnight to ensure good transfer of DNA to the membrane.

After blotting, the apparatus was dismantled and the positions of the wells on the membrane marked with a needle. The membrane was peeled away and the top left hand corner cut for orientation. Finally the membrane was washed in 2XSSC for 15min. to remove the alkaline buffer before hybridising with a radio-labelled cDNA or oligonucleotide probe.

3.8 Preparation of radio-labelled probes

3.8.1 Isolation of cDNA insert from plasmid vector

Approximately 1µg of plasmid DNA was digested with an appropriate restriction enzyme in order to release the cDNA insert. Following RNAse A treatment, the cDNA was separated from the plasmid on a 0.8% low melting point (LMP) agarose gel. The gel was poured into a mini-submarine gel tray at 4°C and allowed to set for 30min. The gel was run in 1XTBE buffer at 4°C and a low voltage (30-50V) to prevent the LMP gel melting and to ensure good resolution of DNA fragments. The gel was

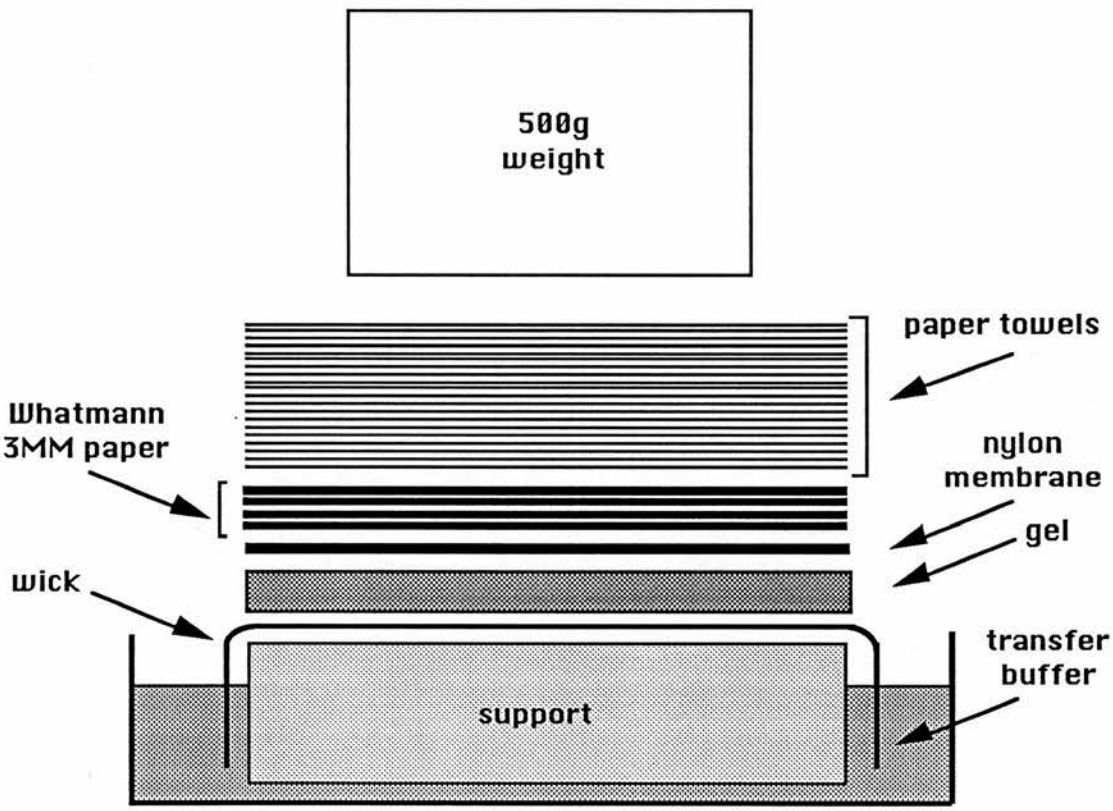


Fig. 3.3

Capillary transfer of DNA from agarose gel to solid nylon membrane. Transfer buffer is drawn from the reservoir through the gel, via the wick, and into a stack of paper towels. The DNA is eluted from the gel by the moving stream of buffer and is deposited on to the nylon membrane. The weight applied to the top of the paper towels ensures a tight connection between the layers of material used in the transfer system.

viewed under u/v light to visualise the insert which was cut out of the gel with a scalpel and placed in a 1.5ml eppendorf tube.

3.8.2 Purification of cDNA insert

The DNA was isolated from the gel slice using Bio-Rad's 'Prep-a-Gene DNA purification matrix' according to manufacturer's instructions.

3.8.3 Random-primer labelling of double-stranded cDNA probes

The cDNA insert was labelled with alpha ^{32}P dCTP (Amersham) by the random primer method based on the method developed by Feinberg and Vogelstein, (1983, 1984), using the Random Primed DNA Labelling Kit (Boehringer) according to manufacturer's instructions. Generally, 50ng of DNA was boiled to denature the double-stranded probe and placed on ice to prevent re-annealing. Label mix containing 1µl each of dGTP, dATP and dTTP dinucleotides and 2µl of reaction mix was prepared and added to the denatured probe, together with 50µCi of radio-isotope and 2µl of Klenow enzyme to a total volume of 20µl. The probe was incubated at 37°C for an hour and then briefly centrifuged to collect reagents at the bottom of the tube. A NucTrap™ Push Column (Stratagene) was used to separate the probe from unincorporated nucleotides. The specific activity of the labelled probe was measured using in a 1209 Rackbeta liquid scintillation counter (LKB Wallac).

Before adding the probe to hybridisation buffer, it was denatured by boiling for 10 min. and placed on ice. Approximately $1x10^6$ counts/ml of labelled cDNA was used in each hybridisation.

3.8.4 Oligonucleotide Synthesis

Synthetic oligonucleotides (generally 18 to 24mers) were synthesised on an Applied Biosystems PCR Mate Synthesiser, removed from the columns with 1ml of ice-cold ammonia and deprotected overnight at 55°C. The deprotected oligonucleotides were stored at -20°C and purified by ethanol precipitation as required. The concentration of a 1/100 dilution of a purified oligonucleotide was determined by measuring the optical density (OD) of the sample at 260nm in a Kontron Analytical spectrophotometer. An OD_{260} of 1.0 = 20µg/ml DNA. Purified oligonuclotides were aliquoted and stored at -20°C.

3.8.5 End-labelling of oligonucleotide probes

Fresh gamma ^{32}P-dATP (Amersham) of high specific activity (10μCi/μl) gave the best results when labelling oligonucleotides. Synthetic oligonucleotides are synthesised without a phosphate group at their 5' termini, and can therefore be easily labelled by transfer of the gamma ^{32}P from [gamma ^{32}P]ATP using the enzyme bacteriophage T4 polynucleotide kinase. The reaction was performed with T4 polynucleotide kinase and 1X kinase buffer in a total volume of 20μl, and incubated at 37°C for 30min. The reaction was terminated by adding 1μl of 0.25M EDTA (pH 8.0). Unincorporated radionucleotides were removed by passing the labelling reaction through a NucTrap™ column according to the manufacturer's instructions. The specific activity of the probe was measured as for cDNA probes. Approximately 5x10^5 counts/ml of labelled oligonucleotide were used in each hybridisation.

3.9 Polymerase chain reaction

Specific regions from clones of interest were amplified using PCR. PCR allows amplification of DNA segments *in vitro* by a succession of incubation steps at different temperatures (Saiki et al., 1985, Mullis et al., 1986, Mullis and Faloona, 1987).

The double stranded DNA is initially denatured (94°C, 3 to 5min., 1 cycle), the DNA is then melted (94°C, 30s.) and two oligonucleotide primers complementary to the 5' and 3' boundaries of the target sequence are annealed at low temperature (Tm -5°C, 1min.) and then extended at an intermediate temperature (72°C, 1min.). These 3 steps are repeated for 30 cycles with a final extension at 72°C for 10min. Programs were designed according to guidelines laid out in 'PCR Protocols' (Innis et al., 1990) and Perkin Elmer Cetus (GeneAmp® PCR Reagent Kit). This process can generally amplify DNA sequences by at least 10^5 fold.

PCR was performed using Taq polymerase (Promega), sense and antisense synthetic oligonucleotide primers synthesized as described in Section 3.8.4 and a programmable heating block (Perkin Elmer Cetus DNA Thermal Cycler).

Reactions were carried out in a total volume of 100μl, which included the following: 200μM of each of the deoxynucleoside

triphosphates dATP, dCTP, dTTP, dGTP, 1X reaction buffer (10mM Tris-HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin), 1μM each of the sense and antisense PCR primers, 2.5U Taq polymerase, 1ng DNA template and sterile water. The reactions were overlaid with 100μl of mineral oil to reduce evaporation. PCR amplification products were visualised under u/v following electrophoresis on 1% (w/v) agarose gels.

3.9.1 Purification of PCR products

PCR amplification products were purified through CHROMA SPIN™ columns (Clontech) according to manufacturer's instructions.

3.10 DNA Sequencing

3.10.1 Manual sequencing - preparation of labelling reactions

Sequencing of genomic DNA, cDNA and PCR fragments was performed according to the dideoxynucleotide chain termination method of Sanger et al. (1977) using the commercially available Sequenase® sequencing kit (U.S.B.). The annealing mix included ~500ng of DNA, 2μl 5X reaction buffer and ~1220ng of oligonucleotide primer. This was boiled for 2min. and placed on ice. Each of the 4 dideoxynucleotides (2.5μl each of ddATP, ddCTP, ddGTP, ddTTP) were prewarmed to 37°C. The labelling mix (1μl) was diluted with 13.5μl of water, and 1μl of the Sequenase™ enzyme was diluted in 7μl of ice-cold enzyme dilution buffer. (10% (v/v) DMSO was added to each of the ddNTPs and to the labelling mix for sequencing of purified PCR fragments).

The labelling reaction was carried out by adding 1μl DTT (0.1M), 2μl diluted labelling mix, 0.5μl ^{35}S dATP (Amersham) and 1.5μl diluted Sequenase™ enzyme to the reaction mix followed by 5min. incubation at room temperature. Reactions were terminated by adding 3.2μl of the sequencing reaction to each of the prewarmed ddNTPs, 4μl of formamide stop dye and incubating at 37°C for 5min. Terminated reaction products were stored on ice.

3.10.2 Manual sequencing - preparation of sequencing gel

Sequencing reaction products were run on Bio-Rad Sequi-Gen™ Sequencing Gel Apparatus. The gel base (27.8g urea, 12ml 30% (w/v)

Acrylamide [19:1] Bisacrylamide Solution (NBL), 15ml 10XTBE, 6ml water) was heated in a microwave to melt the urea, and 150µl of ammonium persulphate (Bio-Rad - 0.1g in 400µl water) and 150µl TEMED (Bio-Rad) added. The gel base was poured immediately into the casting tray to seal the bottom of the gel apparatus. The sequencing gel was made up of top mix (27.6g urea, 12ml acrylamide, 6ml 10XTBE, 20ml water) and bottom mix (9.2g urea, 4ml acrylamide, 5ml 10XTBE, 2ml water), with 60µl each of TEMED and ammonium persulphate added to top mix and 20µl each to bottom mix, once the urea had been melted. A 25ml pipette was used to draw up 7.5ml top mix followed by 6.5ml bottom mix to form the base of the gel. The rest of the top mix was poured down the middle of the gel apparatus and left to polymerise with the gel comb placed upside down. Once polymerised, the comb was removed and the top of the gel washed with 1XTBE.

Gels were run using a Bio-Rad Computer Controlled Electrophoresis Power Supply (Model 3000Xi), in 1XTBE buffer at 1800 volts for 30min. to prewarm. The comb was inserted with the teeth just entering the gel and the wells washed again with 1XTBE. Samples were preheated at 70°C for 2min., loaded and run for 2 to 3 hours at 1800 volts, or overnight at 600 volts. The gel was transferred to Whatman 3MM filter paper, covered with cling-film and dried under vacuum at 80°C for 2 hours on a Savant SGD4050 gel drier (Stratech Scientific). The cling film was removed from the dried gel which was exposed to X-OMAT™ AR Kodak diagnostic film (20cmx25m roll) overnight. The film was developed as described in section 3.2.6.

3.10.3 Automated sequencing

Automated sequencing was performed using an Applied Biosystems 373A DNA sequencer according to the manufacturer's instructions. The *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit uses four ABI dye-labelled dideoxynucleotides: G, A, T and C DyeDeoxy™ Terminators which allow the incorporation of a dye-label, or fluorochrome, into the DNA together with the terminating base. Thermal cycling of the sequencing reactions in a Perkin Elmer Cetus Thermal Cycler increases signal intensity and thus decreases sensitivity to reaction conditions. All four reactions are performed in one tube as different dyes are used which emit light at

different wavelengths when excited by a laser. The emission peaks overlap and have to be resolved by computer analysis.

3.10.4 Sequence Analysis

Sequence analysis was performed using the computer program, GeneJockey, produced by P. L. Taylor, distributed by Biosoft and run on Apple Macintosh computers.

4 Human TRH receptor cDNA: Cloning, functional characterisation and chromosomal localisation.

4.1 Introduction

Thyrotrophin-releasing hormone (TRH), a hypothalamic tripeptide releasing factor, is involved in stimulating the release of thyrotrophin (TSH) from thyrotrophs and prolactin from lactotrophs in the anterior pituitary (Jacobs et al., 1971; Vale et al., 1977; le Dafniet et al., 1983; Gershengorn, 1985). TRH also has several effects in the central nervous system (CNS) including reversal of the narcosis induced by some CNS depressants, the modification of neuronal firing rate and the stimulation of locomotor activity, for reviews see Jackson (1982), Sharif (1985) and Scanlon & Hall (1989). The diverse functions of TRH have been discussed in Chpt. 2.3 (see Table 2.1).

The use of TRH as a potential therapeutic agent in neuronal and endocrine disease prompted the study of its receptor. The design of TRH analogues specific to a particular neuronal pathway, or cell type, in the anterior pituitary would be greatly facilitated by a knowledge of the ligand binding site and an understanding of TRH/receptor interactions and methods of intracellular signalling. It is also particularly important to determine the sequences of G-protein-coupled receptors (GPRs) as mutations in GPRs have been implicated in disease pathologies such as the constitutively activating mutations mentioned in Chpt. 2.6.30. Thus it is vital to be able to compare the sequence and structure of a receptor implicated in a particular pathology with that of the wild-type receptor.

The cloning of the mouse TRH-R (Straub et al., 1990) and rat TRH-R (Sellar et al., 1993) indicated species differences at the carboxy (COOH) tail, a domain implicated in GPR desensitisation and downregulation (Chpt. 2.6.25-29). One of the major problems in clinical medicine involves the desensitisation of GPRs following prolonged exposure to certain drugs and, as a result, much attention has been focused on this GPR domain. The aim of this project was to determine whether the human TRH-R differed from the mouse and rat receptors at the COOH tail and to examine the possibility of the existence of multiple

TRH-R subtypes. The chromosomal localisation of the human TRH-R gene was also undertaken to determine whether the TRH-R gene was linked to any chromosomal abnormalities.

Early studies with the TRH-R in the brain and pituitary indicated similar ligand binding and biochemical properties (Burt & Taylor, 1980). However, the development of synthetic TRH analogues, specific for the behavioural CNS effects of TRH, but lacking TSH-releasing activity (Szirtes et al., 1984), suggested differences in TRH-Rs from these tissues. Johnson et al., (1989) then undertook a comparison of the molecular properties of TRH-Rs in brain and pituitary. Some variation in charge characteristics between solubilised brain and pituitary receptors was observed, possibly reflecting amino acid differences, or variation in post-translational modifications, such as glycosylation or phosphorylation. They also presented evidence that TRH-Rs in the amygdala, septum and hippocampus mediated reduction in narcosis, whereas those in the nucleus accumbens enhanced dopamine release and locomotor activity. Taken collectively, these studies suggested that different TRH-R subtypes might exist.

The transmembrane signalling systems activated by TRH have been intensively studied in the pituitary. TRH has been shown to act via the inositol phospholipid-calcium-protein kinase C pathway (Gershengorn, 1985, 1986) by coupling to a G-protein that activates the second messenger, phospholipase C (see Chpt. 2.6.7-9). Thus the TRH-R was thought to belong to the superfamily of seven transmembrane, GPRs (see Chpt. 2.6). Some controversy exists over the potential involvement of cAMP in TRH signal transduction and, in general, evidence has suggested that cAMP plays a secondary role in transducing the effects of TRH (Denef, 1988). Recently, however, it has been reported that TRH-Rs can couple to both adenylate cyclase (Paulssen et al., 1992) via G_s and phospholipase C via G_q/G_{11} (Hsieh & Martin, 1992) in pituitary GH3 cells, providing further hints that multiple TRH-R subtypes might exist.

Although the pharmacology of TRH binding sites and the biochemistry of its signalling systems has been studied, little was known about the molecular nature of the receptor. Purification of the receptor and the cloning of the TRH-R gene(s) was needed to further the understanding of these processes. The receptor had not been purified, but

rapid advances in recombinant DNA technology since the mid-1980s have resulted in the isolation of cDNAs encoding TRH-Rs from several species.

The first TRH-R to be cloned was a mouse pituitary TRH-R cDNA isolated from thyrotrophic TtT tumour cells using the *Xenopus* oocyte expression system (Straub et al., 1990). Structural characterisation indicated a single polypeptide of 393 amino acids with the predicted similarity to other members of the GPR superfamily (Probst et al., 1992 for review). Following the identification of the mouse TRH-R, several rat TRH-R subtypes were rapidly cloned and characterised. One rat subtype was identified both from a mammotroph-derived GH_4C_1 cell line (Zhao et al., 1992) and from a mammotroph/somatotroph GH_3 tumour cell line (de la Peña et al., 1992a). The rat TRH-R had high sequence homology to the mouse receptor, but encoded 412 amino acids (TRH-R_{412}) due to an extra 19 amino acids at the 3' end of the carboxy (COOH) tail. Other forms of the rat TRH-R were then identified, including a receptor of 387 residues, TRH-R_{387} (de la Peña et al., 1992b) and one of 411 residues, TRH-R_{411} (Sellar et al., 1993). The amino acid variation in both of these subtypes occurred at the COOH terminus. TRH-R_{387} had a 52bp deletion, due to the presence of a retained intron (Breitbart et al., 1987) in the coding sequence of the receptor. Splicing of this 'intron' resulted in a frame-shift that produced 12 variant COOH amino acids. The TRH-R_{411} subtype had a deletion of 3 nucleotides, resulting in the loss of a single amino acid at position 393.

This chapter reports the isolation of a functional human pituitary TRH-R (Duthie et al., 1993a) displaying high sequence homology with the mouse and rat TRH-R sequences, but with amino acid variation at the COOH tail, and discusses the potential significance of this finding in relation to other TRH-R subtypes isolated.

4.2 Materials and methods

This section provides only a brief outline of the methods used to isolate and sequence the human TRH-R. A detailed description of the procedure involved in isolating genes from commercially prepared DNA libraries has been described in Chapter 3. Specific information relevant to the

subcloning, functional analysis and chromosomal localisation studies of the human TRH-R cDNA is included here.

4.2.1 Screening a human λgt10 cDNA library

C600 hfl (high frequency of lysogeny) *Ecoli* host plating cells (Clontech) were innoculated with approximately $1x10^6$ pfu of a λgt10 human pituitary 5' stretch cDNA library (Clontech). The library was probed with a 2.5kb rat TRH-R cDNA fragment (Sellar et al., 1993) labelled with ^{32}P by random primer labelling as described in Chpt. 3.

Positive plaques were isolated following a secondary screening, and the bacteriophage DNA was purified (Chpt. 3.3) and digested with EcoR1 to release the insert (Chpt. 3.7). The size of the insert was also determined by PCR analysis (Chpt. 3.9) using commercially available vector primers (Clontech) designed to amplify the λgt10 insert.

EcoR1 digests of putative clones were Southern blotted (Chpt. 3.7.4) following separation of the restriction fragments by electrophoresis on agarose gels. The blots were again probed with the ^{32}P-labelled 2.5kb rat TRH-R cDNA, to confirm that the isolated plaques hybridised positively to the TRH-R sequence.

4.2.2 Sequencing of positive clones

Sequencing of the purified bacteriophage DNA was carried out several times in both orientations using the automated ABI DNA sequencer (Chpt. 3.10). Initial sequencing primers used were the 5' and 3' vector primers T7 and SP6. Subsequently, oligonucleotides were designed to internal sequence and used as sequencing primers. Sequence analysis was performed using the program GeneJockey on Apple Macintosh computers.

4.2.3 Subcloning the human TRH-R cDNA into pCR™II

The human TRH-R λgt10 clone had an EcoR1 site in the 5'UTR, so the 2.3kb insert could not be released as a single fragment from the vector's EcoR1 cloning site. It was decided therefore, to subclone a PCR-amplified fragment of the receptor into a plasmid vector which could be more easily manipulated than a λ vector. The coding region of the TRH-R from -18bp to 1238bp was first amplified by PCR using the following oligonucleotide

primers (sense primer 5'Ti [nucleotides -18 to +4] 5'dAGCTTCAATCCACTGAAGATGG3' and antisense primer 3'Tp [nucleotides 1217 to 1238] 5'dTTCTCAATTTCTTTGTCATCC3') were based on sequence analysis of the human TRH-R clone as described in section 4.2.2. The 1.2kb PCR product was subcloned directly into the TA cloning vector pCR™II (Invitrogen) as described in Chpt. 3.4.

Several positive colonies were picked and analysed by plasmid lysis PCR (Chpt. 3.5) and each colony was also grown up overnight in 10ml LB broth, supplemented with Amp (50μg/ml), in a 37°C shaking incubator. Colonies that contained the correct size of insert (1.2kb), as detected by plasmid lysis PCR, were purified from the overnight cultures using the Promega Magic Mini-Prep kit (Chpt. 3.6). Purified plasmid DNA was also sequenced to confirm that the pCR™II vector did contain the human TRH-R insert and that no mismatches had been introduced into the subclone as a result of PCR amplification of the original λgt10 human clone.

4.2.4 Chromosomal localisation of the human TRH-R gene

The chromosomal localisation for the human TRH-R gene was achieved by fluorescence *in situ* hybridisation (F.I.S.H.) using the human TRH-R cDNA as a probe. Both the λgt10 human TRH-R cDNA clone (2.3kb) and the 1.2kb human TRH-R subclone in pCR™II were biotinylated and hybridised to metaphase chromosome spreads obtained from normal human males. Hybridisation was detected by incubation with avidin-fluorescein isothiocyanate and the signal amplified twice by two further incubations with biotinylated anti-avidin. Chromosomes were counterstained and examined under a fluorescence microscope. This experiment was conducted in collaboration with Professor J M Connor's group at the Duncan Guthrie Institute of Medical Genetics in Glasgow and a detailed description of the method is provided in Appendix III.

4.2.5 Subcloning the human TRH-R into pcDNA-1

Functional analysis of the human clone required expression of the cDNA in a mammalian cell line. To achieve this, the human TRH-R cDNA in pCR™II was subcloned into the EcoR1 site of the mammalian expression vector pcDNA-1 (Invitrogen) in front of the cytomegalovirus promotor

(CMV) see Fig. 4.1. The following protocol was derived from Sambrook et al., (1989). The human TRH-R pCR™II subclone was digested with EcoR1 to release the 1.2kb insert. The vector and insert were separated by electrophoresis on a 1% LMP agarose gel and the insert was purified from the LMP gel (Chpt. 3.8.2) and concentrated to ~50ng/µl by ethanol precipitation.

A total of 5µg of pcDNA-1, which has both Tet and Amp resistance genes, was also digested with EcoR1 in 5 x 1µg aliquots. A sample of each digest was electrophoresed on a 1% mini-agarose gel to ensure that the plasmid had been completely linearised. Once digestion was complete, the samples were pooled, making up a total volume of 100µl, phenol/chloroform extracted, precipitated for 15min. at -20°C and resuspended in 10mM Tris/HCl pH8.3.

Vector dephosporylation: To prevent the plasmid from self-ligating, the 5' phosphate groups were removed by digestion with calf intestinal alkaline phosphatase (CIAP) (Promega) for 30min. at 37°C in a total volume of 100µl:- 60µl linearised pcDNA-1, 1µl CIAP (1U/100pM 5' terminal phosphate residues), 10µl 10X CIAP buffer, 29µl water. After dephosphorylation, the enzyme was denatured with 0.5% SDS and 5mM EDTA and proteinase K added to a final concentration of 100µg/ml. The reaction mix was incubated at 56°C for 30min. and cooled to room temperature. It was then extracted once with phenol and once with chloroform before ethanol precipitation with 3M sodium acetate, pH7 (EDTA precipitates out of solution at acid pH) for 15min. The pellet was washed in 70% ethanol and resuspended at a concentration of 100µg/ml in TE buffer

Ligation reactions: The following ligations were set up and incubated at 16°C overnight: A - vector + insert (1:1 molar ratio); B - vector + insert (1:3 molar ratio); C - vector only; D - insert only; E - no DNA. The ligation reactions were performed in a total volume of 30µl with 3µl T4 DNA ligase (3U/µl) (Clontech, Union-DNA ligation kit), 3µl 10X ligation buffer, and 3µl rATP (10mM).

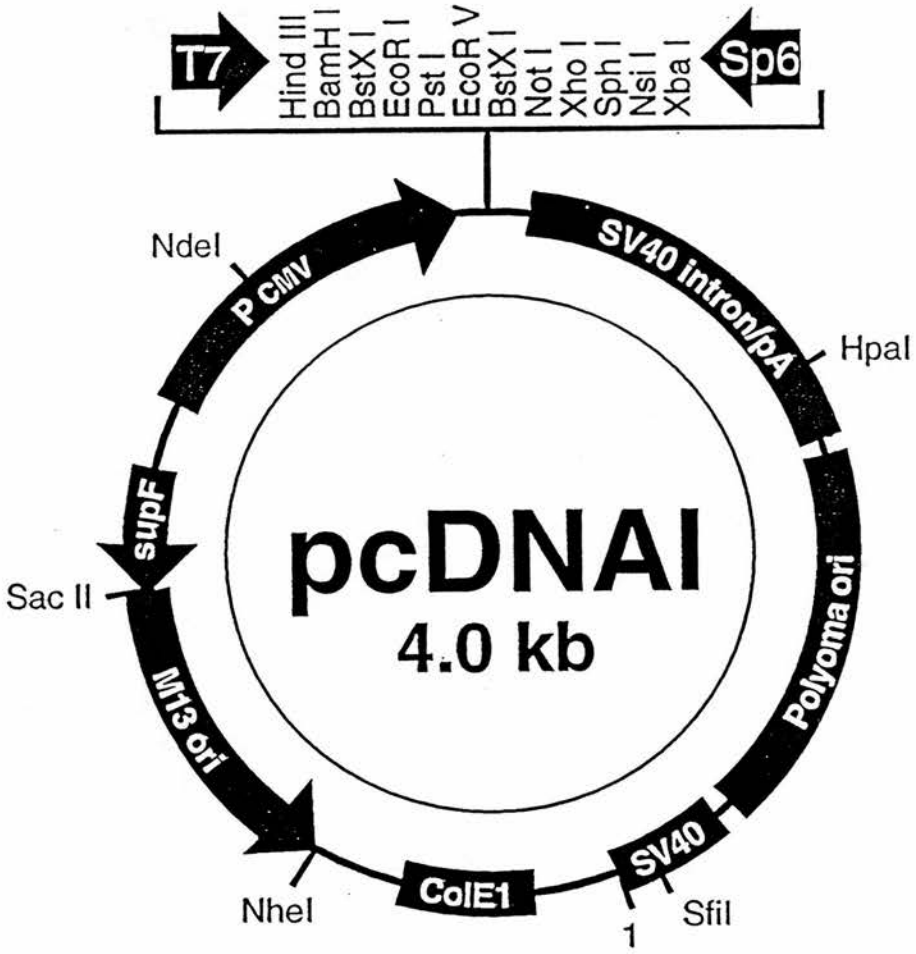


Fig. 4.1

The mammalian expression vector pcDNA-1.

High efficiency electro-transformation of *E.coli*: MC1061/P3 electrocompetent cells (Invitrogen) were transformed with the ligated DNA using a Bio-Rad Gene Pulser set at 25μF and 2.5kV with the Pulse Controller at 200 Ohms. Aliquots of electrocompetent cells (40μl) were thawed carefully on ice. Between 1μl and 2μl of ligation mix was added to each 40μl cell suspension, mixed gently and incubated on ice for 1min. before being transferred to a cold 0.2cm sterile electroporation cuvette (Bio-Rad) and pulsed for 15 seconds. An aliquot of the MC1061/P3 cells was also transformed with 10ng of supercoiled pcDNA-1 as a positive control. The transformed cells were immediately resuspended in 1ml SOC medium (Appendix I) and incubated at 37°C with vigorous shaking. After 1 hour's incubation, colonies were selected by plating 100μl of the SOC mixture onto LB agar plates containing Tet (12.5μg/ml) and Amp (50μg/ml) and incubated overnight at 37°C.

Colonies that grew on Amp/Tet plates were picked and subjected to plasmid lysis PCR and were also grown up as overnight cultures in 10ml LB broth (50μg/ml Amp) and purified using the Promega Magic Mini-Prep kit. To determine the orientation of the TRH-R insert within the vector, an internal PCR oligonucleotide primer (5'Tx [nucleotides 829 to 849] 5'dCTTTTATGGATGCCCTACAGG3') was used in conjunction with the vector primer SP6 to amplify a small region of the insert. Subclones were sequenced to confirm the orientation of the TRH-R insert.

4.2.6 Transient transfection of COS-1 cells

Tissue culture work was performed in a sterile Class II hood with all media and solutions prewarmed to 37°C in a water bath. Monolayer cultures of COS-1 cells ($5x10^5$ cells/100mm dish) were plated out in 5ml of DMEM medium (Appendix I) in 100mm dishes and allowed to attach overnight at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air.

Transient transfections were performed using LIPOFECTIN® Reagent (GIBCO-BRL). LIPOFECTIN is a formulation of cationic lipids which, when mixed with negatively charged DNA, forms a neutral lipid-DNA micelle complex (Felgner et al., 1987). Micelles fuse with the cell membrane and discharge their contents into the cell.

For each transfection, human TRH-R in pcDNA-1 (3μg) and LIPOFECTIN Reagent (50μg) were each pre-diluted in 1.5ml serum-free

DMEM medium in sterile polystyrene jars (Greiner). Antibiotics and serum inhibit the action of LIPOFECTIN. Diluted DNA and LIPOFECTIN Reagent were combined, mixed gently, and incubated at room temperature for 15min. This predilution step should prevent any precipitate forming.

Monolayer cultures of COS-1 cells (60 to 70% confluent) were washed (X2) with 5ml of serum-free medium. The diluted DNA/LIPOFECTIN mix (3ml) was added to each dish and incubated for 4hrs at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air. The DNA-containing medium was then replaced with 10ml of complete DMEM medium and the cells left overnight to recover from the transfection procedure. Transfected cells were assayed for the presence and activity of receptors 72hrs post transfection.

4.2.7 Inositol phosphate (IP) production assay

COS-1 cells were trypsinised 24 hours after transient transfection with the human TRH-R pcDNA-1 subclones and transferred to 12-well plates. Cells were labelled to isotopic equilibrium with 2μCi/ml myo-^{3}[H]inositol (Amersham) in inositol-free DMEM medium (Appendix I).

This medium was removed 72hrs post-transfection and 270μl Buffer A (Appendix I) containing LiCl (10mM) was added to each well and incubated at 37°C for 15min., (LiCl inhibits IP1 phosphatase, an enzyme involved in the degradation of IP1 to inositol). TRH concentration (30μl of 10^{-5}M) was added to the wells in triplicate and the cells returned to the incubator for 45min.

The drug-containing medium was removed and the reaction terminated by adding 500μl 0.5M perchloric acid (PCA) solution containing 5mM EDTA (PCA/EDTA stop solution) to the cells, followed by 50μl phytic acid (1.8mg/ml) and incubating for 10min. at 4°C. The reaction mixture was transferred to numbered test tubes, and neutralised with 0.5mM potassium hydroxide/60mM Hepes, containing Universal pH indicator, producing a 'mid-green' colour (pH 7-7.5).

Cell extracts were centrifuged for 15min. at 3000rpm and 900μl of the resulting supernatant was removed and added to test tubes containing 500μl Dowex resin (Analytical grade anion exchange AG® 1-X8 Resin,

100-200 mesh, Bio-Rad), gently mixed with a whirlimixer, allowed to settle and the supernatant removed by aspiration. Inositol, glyceroinositol phosphates and total inositol phosphates (containing IP1, IP2 and IP3) fractions were eluted from the resin by washing sequentially with 1ml H_2O, 60mM ammonium formate/5mM sodium tetraborate and 1M ammonium formate/0.1M formic acid respectively. An aliquot (800µl) of the total IP fraction was added to 5ml Optiphase HP (Pharmacia) liquid scintillant and the radioactivity measured in an LKB Rackbeta liquid scintillation counter.

4.2.8 Measurement of total cell radioactivity

Total cell radioactivity was measured to compensate for differences in cell number between wells. Wells were washed twice with 500µl of the PCA/EDTA stop solution and solubilised with 500µl 0.1M NaOH solution. The resultant solution was transferred to scintillation vials and neutralised with 260µl 0.66% acetic acid. Scintillation fluid (4ml) was added to each vial and the radioactivity measured as described previously.

4.2.9 Receptor binding assay

Monolayer cultures of transfected COS-1 cells (section 4.2.6) were washed twice with phosphate buffered saline (PBS) (Flow Laboratories), a further 2ml PBS added and manually harvested by scraping with a cell-scraper (Costar). Whole cell suspensions were transferred to sterile universals (15ml) and centrifuged at 3000rpm for 10min. to obtain cell pellets. The supernatant was carefully poured off, the cell pellet resuspended in 2ml of ice-cold Assay Buffer (Appendix I) and allowed to stand for 10min. on ice to lyse the cells. Cell suspensions were then homogenised with coarse and then fine pestles in a Teflon homogeniser. Using a pasteur pipette, homogenates were transferred to 2ml Eppendorf tubes and centrifuged at 11000rpm at 4°C for 30min. The supernatant was decanted and the prepared cell membranes stored at -70°C until use.

Ice-cold Assay Buffer was added to thawed cell membranes on ice. An even membrane suspension was obtained by aspirating the membranes through a 19 gauge needle. Ligand binding assays were

carried out using the TRH agonist, ^{3}H[3-Me-His^{2}]TRH (Peninsula). The radiolabel was diluted (10^{-3}) in Assay Buffer and 50µl added to each tube. Cold peptide [3-Me-His^{2}]TRH (50µl) was added at varying concentrations ranging from 10^{-10}M to 10^{-8}M, and the reactions performed in triplicate. Finally, 400µl of the cell membranes was added to each tube resulting in a final assay volume of 500µl. In addition, maximal binding (B_o) was measured by replacing the cold peptide with assay buffer, and non-specific binding was then measured by adding a saturating dose (10^{-5}M) of ^{3}H[3-Me-His^{2}]TRH.

Following incubation on ice for 60min., the membranes were filtered using a Brandel Cell Harvester (SEMAT). Filter paper (GF/B Whatman) was soaked in Assay Buffer containing 1% polyethylene glycol (Sigma), a detergent that prevents the radiolabel from sticking to the apparatus. The filters were washed three times in rapid succession, with Assay Buffer, to separate the free ligand from ligand bound to the cell membranes. Numbered scintillation vials were placed on a tray, a dried filter circle placed in each vial and 4.5ml scintillation fluid added. The vials were placed on a shaker overnight to allow the radioactivity to leach out of the filters, before counting on a Rackbeta liquid scintillation counter.

4.2.10 Calcium imaging

This experiment was performed in collaboration with Dr Lorraine Anderson and a detailed description of the method used can be found in Appendix III. COS-1 cells, transiently transfected with the human TRH-R were loaded with the fluorescent calcium dye, fura-2 AM (4µM). Intracellular calcium ($[Ca^{2+}]_i$) was then measured in single cells stimulated with 10^{-6}M TRH, using dual wavelength fluorescence microscopy combined with dynamic video-imaging.

4.3 Results

4.3.1 Human TRH-R cDNA: cloning and structural analysis

One positive clone was isolated from the λgt10 pituitary cDNA library. This clone contained an insert of approximately 2.3kb. Sequencing of the positive clone indicated the largest open reading frame (ORF) as being

1194bp, and the ATG initiation codon was identified by comparison with the mouse and rat TRH-R sequences. The nucleotide and deduced amino acid sequences for the human receptor are shown in Fig. 4.2. The human TRH-R encoded a putative GPR protein of 398 amino acids (Fig. 4.3), as compared to 393 for the mouse TRH-R (Straub et al., 1990), and 412 (Zhao et al., 1992; de la Peña et al., 1992a), 387 (de la Peña et al., 1992b) and 411 amino acids (Sellar et al., 1993) for the rat TRH-Rs.

The deduced amino acid sequence has several possible consensus sites for modification of the receptor protein, including sites for potential N-linked glycosylation (Asn-X-Ser/Thr) (Kornfeld & Kornfeld, 1985) and phosphorylation (Ser and Thr residues in varying consensus motifs, depending on the kinase) (Kemp & Pearson, 1990, Kennelly & Krebs, 1991) as indicated in Fig. 4.2. The human clone contained approximately 1kb of 5' untranslated region (UTR), mostly unsequenced and only 130bp of 3'UTR. There was no polyadenylation signal present.

Comparison of the human, mouse and rat TRH-R sequences indicated very high homology between the species. The only major sequence variation occurred at the same site in each receptor (residue 392) at the 3' end of the COOH tail (Fig. 4.4). Following residue 392, the human receptor had 6 variant amino acids, the mouse had 1 amino acid and the long forms of the rat receptor had 20 (TRH-R_{412}) and 19 (TRH-R_{411}) residues respectively. The short form of the rat receptor (TRH-R_{387}) had a 52bp deletion in this region which produced a frame-shift and resulted in 12 variant amino acids at the COOH tail.

4.3.2 Chromosomal localisation of the TRH-R gene

Forty-one metaphases from two normal human males were scored following hybridisation to the 2.3kb λgt10 TRH-R probe, and the position of 73 signals recorded. Of these, a highly significant ($p<0.005$) 15 signals (20.5%) were located on chromosome 8 with 13 (17.8%) comprising a signal peak at 8q23.

Forty-four metaphases from two normal males were scored following hybridisation to the 1.2kb pCR™II TRH-R probe, and the position of 186 hybridisation signals recorded as shown in Fig. 4.5. Of these, a highly significant ($p<0.005$) 35 signals (18.8%) were located on chromosome 8 with 26 (14%) comprising a signal peak at 8q23. These

```
-19                                                                  TAGCTTCAAGCCACTGAAG
                ▽                       ▽
    Met Glu Asn Glu Thr Val Ser Glu Leu Asn Gln Thr Gln Leu Gln Pro Arg Ala Val Val Ala Leu   22
  1 ATG GAA AAC GAG ACA GTC AGT GAA CTG AAC CAA ACA CAG CTT CAG CCA CGA GCA GTG GTG GCC TTA

    Glu Tyr Gln Val Val Thr Ile Leu Leu Val Leu Ile Ile Cys Gly Leu Gly Ile Val Gly Asn Ile   44   I
 67 GAA TAC CAG GTG GTC ACC ATC TTA CTT GTA CTC ATT ATT TGT GGC CTG GGC ATT GTA GGC AAC ATC
                                                        o
    Met Val Val Leu Val Val Met Arg Thr Lys His Met Arg Thr Pro Thr Asn Cys Tyr Leu Val Ser   66
133 ATG GTA GTC CTG GTT GTC ATG AGA ACC AAG CAC ATG AGG ACC CCC ACA AAC TGC TAC CTG GTG AGC      II

    Leu Ala Val Ala Asp Leu Met Val Leu Val Ala Ala Gly Leu Pro Asn Ile Thr Asp Ser Ile Tyr   88
199 CTG GCA GTA GCT GAT CTC ATG GTC TTG GTG GCC GCA GGC CTC CCC AAC ATA ACA GAC AGT ATC TAC

    Gly Ser Trp Val Tyr Gly Tyr Val Gly Cys Leu Cys Ile Thr Tyr Leu Gln Tyr Leu Gly Ile Asn  110
265 GGT TCC TGG GTC TAT GGC TAT GTT GGA TGC CTC TGC ATT ACT TAC CTC CAG TAT TTG GGA ATT AAT     III

    Ala Ser Ser Cys Ser Ile Thr Ala Phe Thr Ile Glu Arg Tyr Ile Ala Ile Cys His Pro Ile Lys  132
331 GCA TCC TCT TGT TCA ATA ACA GCC TTT ACC ATT GAG AGG TAC ATA GCA ATC TGT CAC CCC ATC AAA
                o
    Ala Gln Phe Leu Cys Thr Phe Ser Arg Ala Lys Lys Ile Ile Ile Phe Val Trp Ala Phe Thr Ser  154
397 GCC CAG TTT CTC TGC ACA TTT TCC AGA GCC AAA AAG ATT ATC ATC TTT GTC TGG GCT TTC ACA TCT      IV

    Leu Tyr Cys Met Leu Trp Phe Phe Leu Leu Asp Leu Asn Ile Ser Thr Tyr Lys Asp Ala Ile Val  176
463 CTT TAC TGT ATG CTC TGG TTC TTC TTG CTG GAT CTC AAT ATT AGC ACC TAC AAA GAT GCT ATT GTG
                o
    Ile Ser Cys Gly Tyr Lys Ile Ser Arg Asn Tyr Tyr Ser Pro Ile Tyr Leu Met Asp Phe Gly Val  198
529 ATA TCC TGT GGC TAC AAG ATC TCC AGG AAT TAC TAC TCA CCT ATT TAC CTA ATG GAC TTT GGT GTC      V

    Phe Tyr Val Val Pro Met Ile Leu Ala Thr Val Leu Tyr Gly Phe Ile Ala Arg Ile Leu Phe Leu  220
595 TTT TAT GTT GTG CCA ATG ATC CTG GCT ACC GTC CTC TAT GGA TTC ATA GCT AGA ATC CTT TTC TTA
                                                    ▽
    Asn Pro Ile Pro Ser Asp Pro Lys Glu Asn Ser Lys Thr Trp Lys Asn Asp Ser Thr His Gln Asn  242
661 AAT CCC ATT CCT TCA GAT CCT AAA GAA AAC TCT AAG ACA TGG AAA AAT GAT TCA ACC CAT CAG AAC
                ▽                                       o
    Thr Asn Leu Asn Val Asn Thr Ser Asn Arg Cys Phe Asn Ser Thr Val Ser Ser Arg Lys Gln Val  264
727 ACA AAT CTG AAT GTA AAT ACC TCT AAT AGA TGT TTC AAC AGC ACA GTA TCT TCA AGG AAG CAG GTC

    Thr Lys Met Leu Ala Val Val Val Ile Leu Phe Ala Leu Leu Trp Met Pro Tyr Arg Thr Leu Val  286  VI
793 ACC AAG ATG CTG GCA GTG GTT GTA ATT CTG TTT GCC CTT TTA TGG ATG CCC TAC AGG ACT CTA GTG

    Val Val Asn Ser Phe Leu Ser Ser Pro Phe Gln Glu Asn Trp Phe Leu Leu Phe Cys Arg Ile Cys  308
859 GTT GTC AAC TCA TTT CTC TCC AGT CCT TTC CAA GAA AAT TGG TTT TTG CTC TTT TGC AGA ATT TGC
                                                                                          VII
    Ile Tyr Leu Asn Ser Ala Ile Asn Pro Val Ile Tyr Asn Leu Met Ser Gln Lys Phe Arg Ala Ala  330
925 ATT TAT CTC AAC AGT GCC ATC AAC CCG GTG ATT TAC AAT CTC ATG TCC CAG AAA TTC CGT GCA GCC
                                    o                       ▽
    Phe Arg Lys Leu Cys Asn Cys Lys Gln Lys Pro Thr Glu Lys Pro Ala Asn Tyr Ser Val Ala Leu  352
991 TTC AGA AAG CTC TGC AAC TGC AAG CAG AAG CCA ACA GAG AAA CCT GCT AAC TAC AGT GTG GCC CTA
    ▽
    Asn Tyr Ser Val Ile Lys Glu Ser Asp His Phe Ser Thr Glu Leu Asp Asp Ile Thr Val Thr Asp  374
1057 AAT TAC AGC GTC ATC AAG GAG TCA GAC CAT TTC AGC ACA GAG CTT GAT GAT ATC ACT GTC ACT GAC

    Thr Tyr Leu Ser Ala Thr Lys Val Ser Phe Asp Asp Thr Cys Leu Ala Ser Glu Val Ser Phe Ser  396
1123 ACT TAC CTG TCT GCC ACA AAA GTG TCT TTT GAT GAC ACC TGC TTG GCT TCT GAG GTA TCC TTT AGC

    Gln Ser Stop                                                                          398
1189 CAA AGT TGA ATGGAAAACGAGACAGTCAGTGAACTGAACCAAACACAGCTTCAGCCACGAGCAGTGGTGGCCTTAGAATACCAG
1273 GTGGTCACCATCTTACTTGTACTCATTATTTGTGGCCTGGGCATTGTAGGCAACATC
```

Fig. 4.2

Nucleotide and deduced amino acid sequences of the TRH-R clone isolated from human pituitary gland. The putative transmembrane domains I to VII are underlined and are assigned on the basis of a Kyte and Doolittle hydrophobicity plot (1982). Potential N-linked glycosylation sites are indicated by (Δ) and phosphorylation sites by (°).

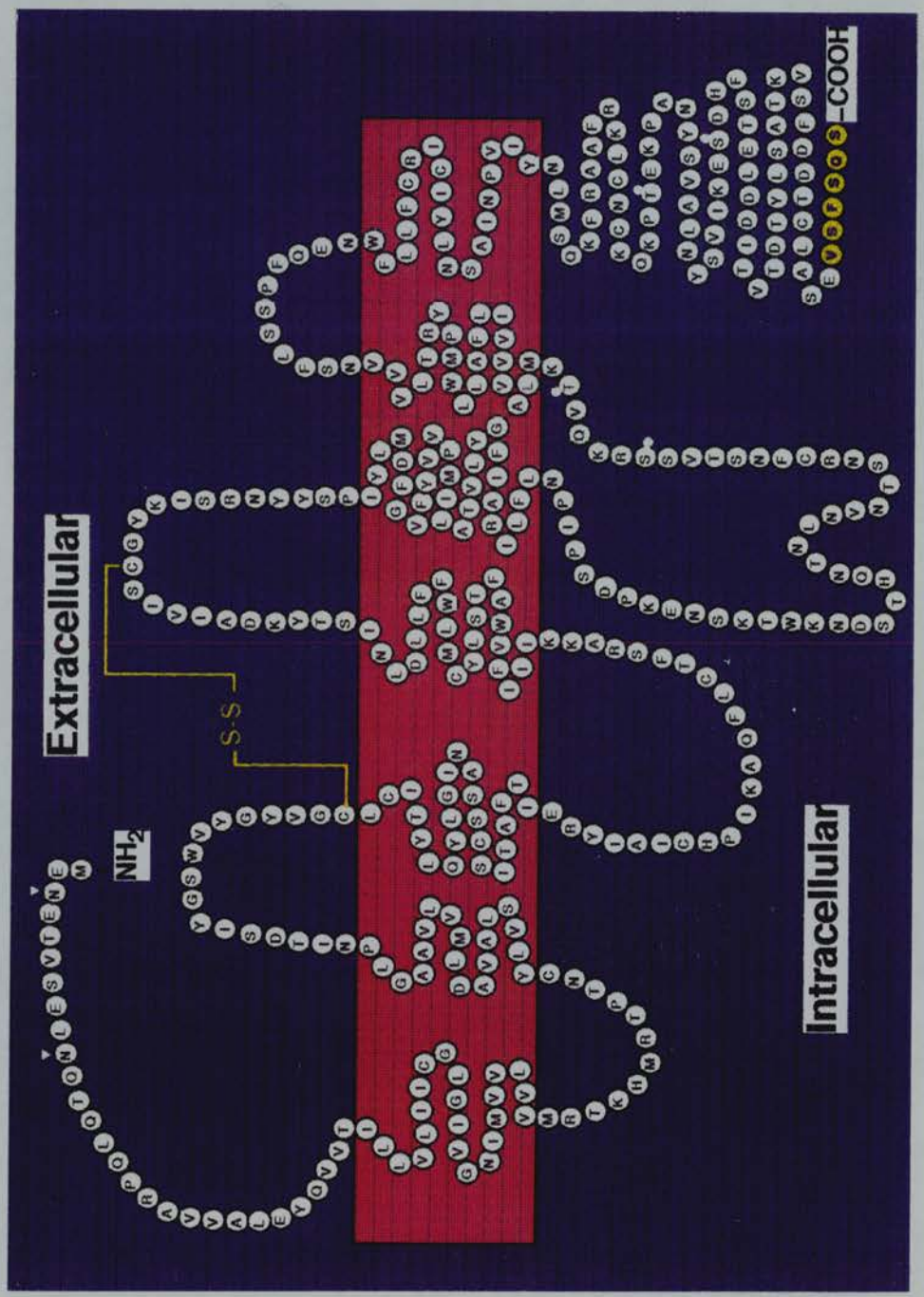


Fig. 4.3

Schematic representation of the proposed seven transmembrane-spanning domains and deduced amino acid sequence for the human TRH-R. Putative di-sulphide bridges between cysteine residues are highlighted in yellow. The alternative COOH tail encoded by the human TRH-R is also highlighted in yellow. Potential N-linked glycosylation sites are indicated by (Δ) and phosphorylation sites by (°).

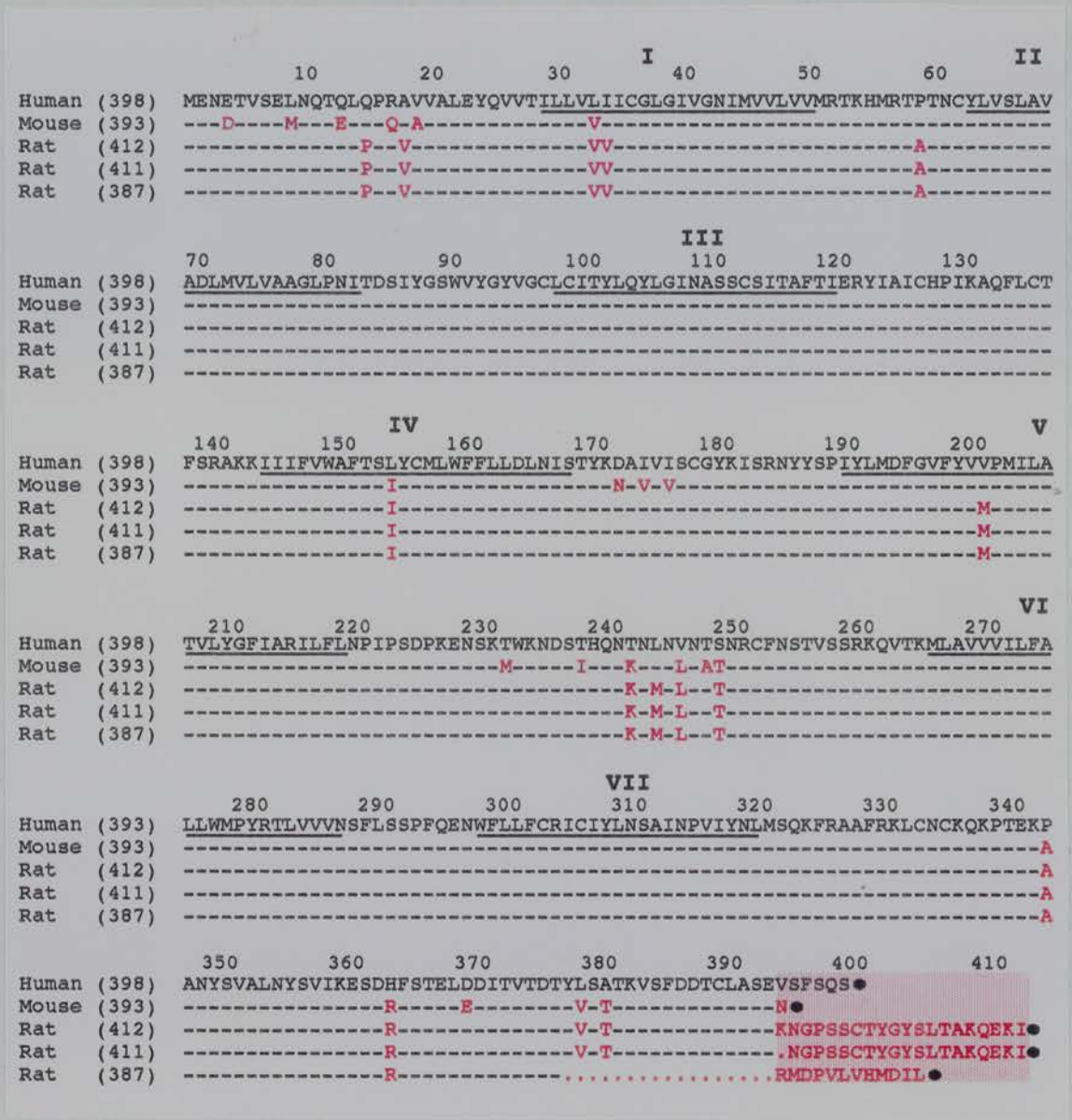


Fig. 4.4

Alignment of the amino acid sequences of human, mouse and rat TRH-Rs. Numbers in brackets after each species name indicates the number of amino acids encoded by each subtype. The amino acid sequence is shown in one-letter code. Homology between the sequences is indicated by dashes. Variant amino acids are indicated in red. Red dots indicate deleted amino acids. Stop codons are indicated by (•), and the putative transmembrane domains are underlined. Aligned sequences are: human (398), mouse (393) Straub et al. [1990]; Rat (412) Zhao et al. [1992], de la Peña et al. [1992a]; rat (411) Sellar et al. [1993]; rat (387) de la Peña et al. [1992b].

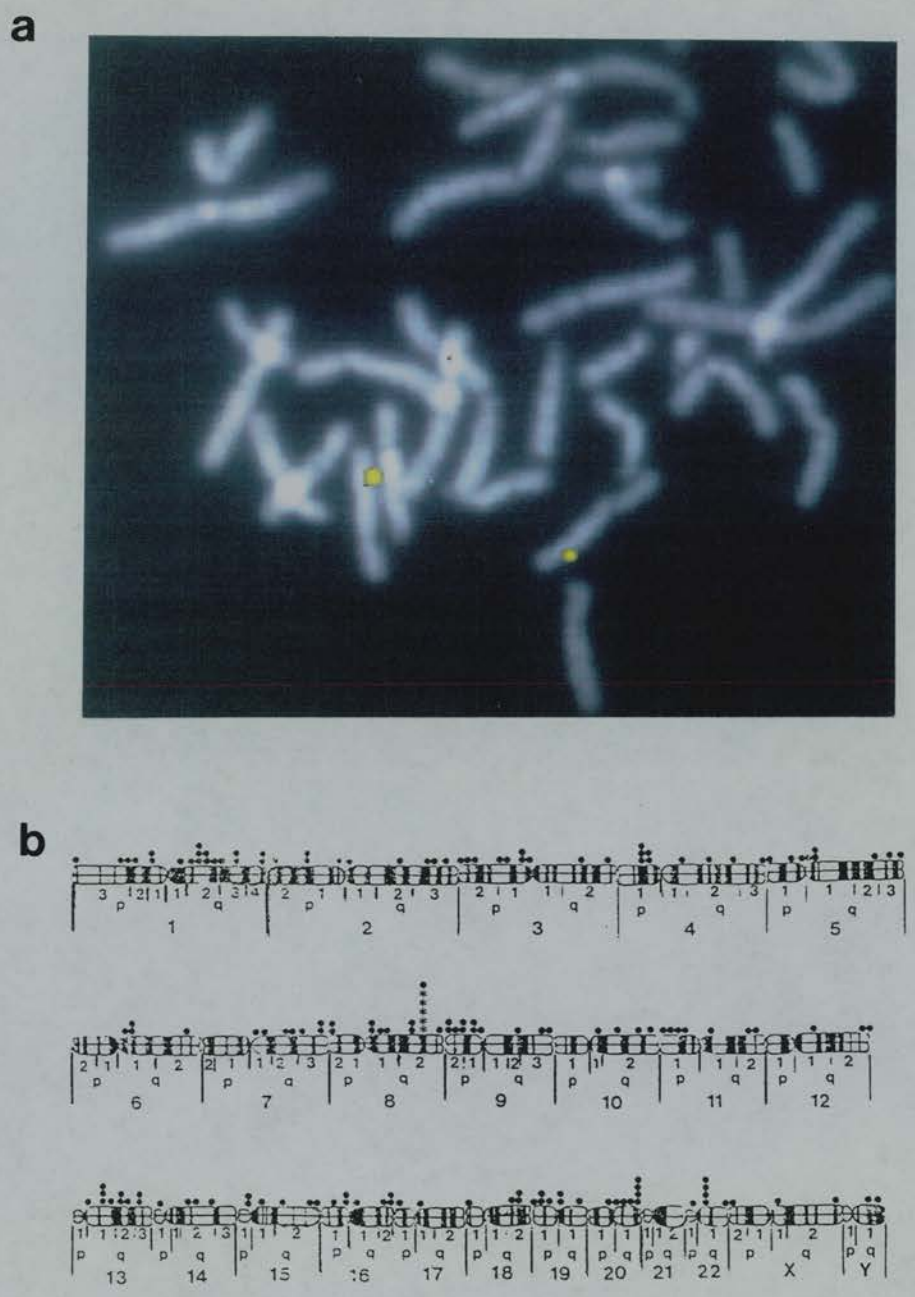


Fig. 4.5

Chromosomal localisation of the human TRH-R gene to chromosome 8q23. Fig. 4.4a illustrates a normal human male partial metaphase spread, hybridisation signals show up as yellow spots. Fig. 4.4b shows signal distribution over 44 normal human male metaphases following hybridisation to the 1.2kb human TRH-R pCR™II probe. (*) represents 5 signals.

experiments support the assignment of the gene for the human TRH-R to chromosome 8q23 (Morrison et al., *in press*).

4.3.3 Human TRH-R expression and functionality

The human TRH-R clone was subcloned into the mammalian expression vector, pcDNA-1, to determine whether it encoded a functional GPR. The binding of TRH to its receptor results in the rapid activation of the stimulatory G-proteins, G_q/G_{11} (Hsieh & Martin, 1992) and subsequently, the activation of the enzyme phospholipase C and the PI second messenger pathway as described in Chpt. 2.6.8.

COS-1 cells were transiently transfected with the human TRH-R in the correct orientation, and following exposure to TRH (10^{-6}M), total IP production was measured and was found to increase approximately twofold. In untransfected cells TRH had no effect (Fig. 4.6). Receptor-ligand binding assays were performed for the human TRH-R in the correct orientation. Scatchard analysis (Fig. 4.7) indicated a single high affinity binding site with an estimated K_d (equilibrium dissociation constant) of 4.28nM and a Bmax (receptor numbers expressed in pmol/mg protein) of 41.8pM/mg.

Functionality of the human clone was further confirmed using calcium image analysis. Activation of the intracellular PI pathway results in the release of intracellular calcium $[Ca^{2+}]_i$ from Ca^{2+} stores within the cell (see Fig. 2.11 in Chpt. 2.6). TRH (10^{-6}M) produced a rapid elevation of $[Ca^{2+}]_i$ in fura-2-labelled, single COS-1 cells expressing the human TRH-R. $[Ca^{2+}]_i$ rose from a resting level of 27nM to a peak of 177nM with levels returning to control values approximately 1.6min. after addition of TRH (Fig. 4.8). In this experiment, only two cells in a field of ten single cells responded to TRH. This was probably due to the low transfection efficiency generally found in transiently transfected, as opposed to stably transfected cells.

The ability of TRH to produce both a rise in total IP production and $[Ca^{2+}]_i$ confirms that the human TRH-R clone, in the direct orientation, behaves as a functional G-protein-coupled receptor with a single high affinity binding site for TRH.

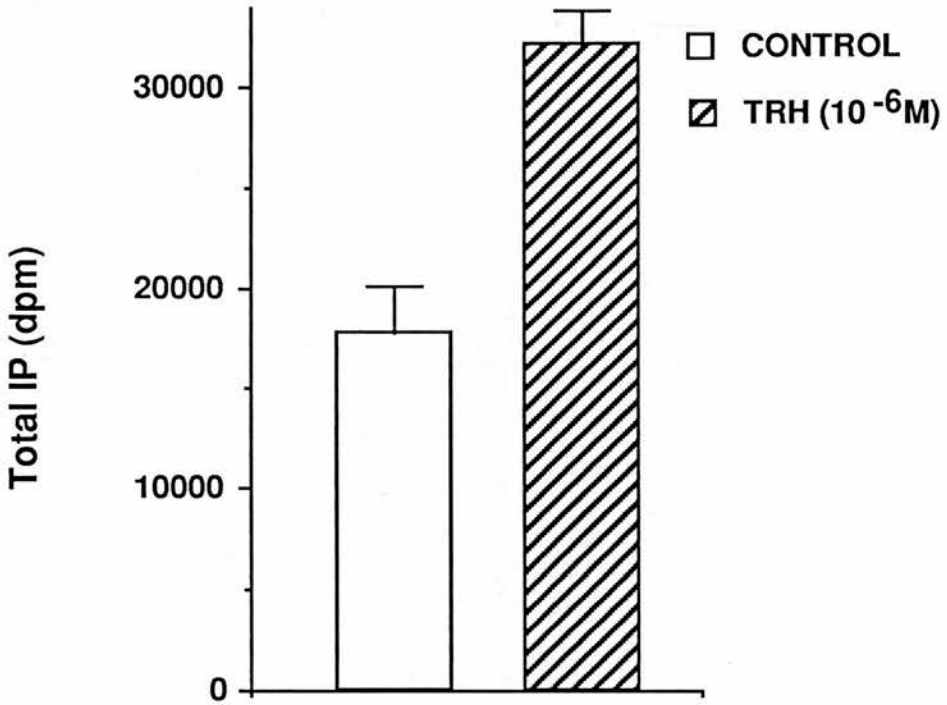


Fig. 4.6

TRH-induced IP production in COS-1 cells transiently transfected with the 1.2kb human TRH-R in pcDNA-1. Twenty four hours after transfection, cells were trypsinised and labelled with myo-[^{3}H]inositol (2μCi/ml for 48 hours) and IP production measured after exposure to TRH (10^{-6}M, 30min.). Results are expressed as the mean ± SEM, n = 3.

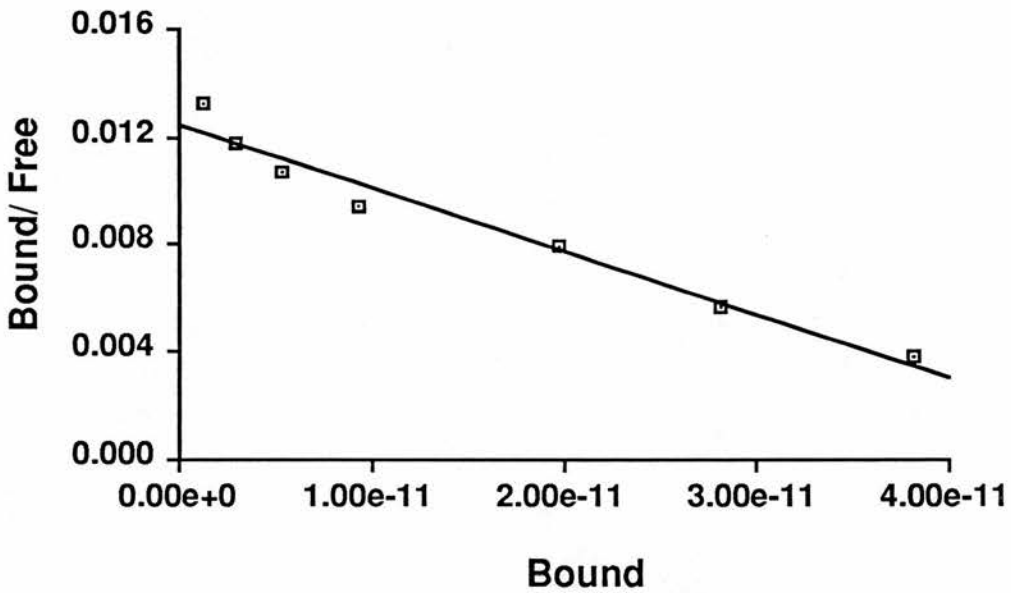


Fig. 4.7

Scatchard plot showing TRH-R binding. Binding studies in COS-1 cells transiently transfected with the 1.2kb human TRH-R pcDNA-1 clone showed the existence of a single-high affinity binding site with a K_d of 4.28nM.

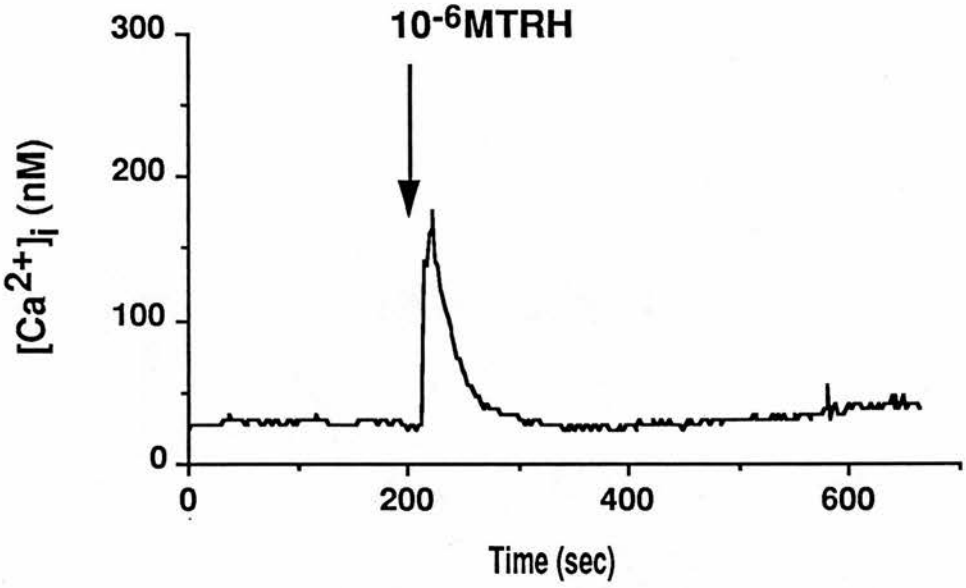


Fig. 4.8

The effect of TRH (10^{-6}M, t = 210sec., n = 2) on intracellular calcium $[Ca^{2+}]_i$ in COS-1 cells transiently transfected with the 1.2kb human TRH-R pcDNA-1 clone.

4.4 Discussion

This chapter has described the isolation and sequencing of a cDNA encoding a human pituitary TRH-R. Evidence that the cDNA encoded a functional, G-protein-coupled TRH-R was obtained from COS-1 cells transiently transfected with the human TRH-R. The binding of a radiolabelled TRH analogue to membranes of transfected COS-1 cells was demonstrated, and the ability of TRH to induce inositol phosphate formation, and the subsequent mobilisation of $[Ca^{2+}]_i$ further confirmed the functionality of the human clone. Finally, sequence comparison of the isolated human clone with the previously reported mouse (Straub et al., 1990) and rat TRH-Rs (Zhao et al., 1992; de la Peña et al., 1992a,b; Sellar et al., 1993) indicated high sequence homology within the coding region of all three species, with major variation occurring only at the distal end of the cytoplasmic COOH tail. The more recently isolated human brain TRH-R (Matre et al., 1993[1]) is identical to the pituitary receptor, but was formed by splicing together two different clones with overlapping sequence homology from TM3 to TM6. An identical human TRH-R was also isolated from a pituitary cDNA library by Yamada et al, (1993[1]). The TRH-R gene structure and the possibilities of alternative splicing are more fully discussed in Chpt. 6.

Primary sequence analysis: The amino acids of the 7 transmembrane (TM) domains were highly conserved between the human, rat and mouse TRH-Rs. Aside from the COOH tail variation, most amino acid changes occurred in the extracellular amino (NH_2) terminus and the third cytoplasmic loop (CL3) when compared to the rat and mouse sequences (Fig. 4.4).

Several residues thought to be of functional importance in GPRs were found to be similarly conserved in the TRH-Rs from all three species. For example, the human TRH-R has two putative consensus sites (Asn-X-Ser/Thr) for N-linked glycosylation (Kornfeld & Kornfeld, 1985) in the NH_2 domain, two in CL3, and two in the COOH tail (Fig.4.2). N-linked

1 The paper by Duthie et al., was accepted for publication by Molecular and Cellular Endocrinology on June 30, 1993. The paper by Matre et al., appeared in BBRC on August 31, 1993 and the paper by Yamada et al., appeared in BBRC on September 15, 1993.

glycosylation is thought to be important for correct insertion of the receptor in the plasma membrane (Chpt. 2.6.22). Disulphide bonds are known to form between Cys residues in the extracellular loops of the β-adrenergic receptor (βAR), thereby stabilising its tertiary structure (Dohlman et al., 1990). A disulphide bond may also occur between similar conserved Cys residues in the extracellular loops (EL) EL1 (Cys^{98}) and EL2 (Cys^{179}) of the TRH-R (Fig. 4.3) as a reduction in TRH-R density occurred when disulphide bonds were disrupted by dithiothreitol (DTT) (Sharif & Burt, 1984). A conserved Cys residue, present in the COOH tail of many GPRs (Cys^{337} in the TRH-R) may be palmitoylated, enabling the formation of a fourth cytoplasmic loop (O'Dowd et al., 1989; Mouillac et al., 1992).

The TRH-R also encodes the highly conserved Asn in TM1 (Asn^{43}) and Asp^{61} in TM2 (Fig. 4.3). These residues are present in nearly all GPRs (Probst et al., 1992) and, therefore, are likely to be involved in the correct folding of the protein for expression in the membrane, which in turn promotes ligand binding and second messenger activation. Indeed, mutation of the rat TRH-R TM2 Asp to Ala (Perlman et al., 1992) resulted in a decrease in ligand binding affinity and abolished TRH-induced stimulation of inositol phosphates, supporting this theory.

An Asp residue found in TM3 of all receptors that couple to cationic ligands, (Strader et al., 1988) is not conserved in the TRH-R, neither are the Ser residues in TM5 which are thought to bond with aromatic hydroxyl groups of catecholaminergic ligands (Strader et al., 1989a; Probst et al., 1992 for GPR sequence alignment). TRH contains a His residue that can be protonated, and it was initially thought that TRH may bind to its receptor via ionic interactions, however, experiments by Perlman et al. (1992) demonstrated reduced TRH binding efficiency at low pH. They also mutated Asp residues in TM2, TM4, TM5 and EL2 that were possible residues for ionic interactions with TRH, and only the Asp in TM2 had any effect on ligand binding, as mentioned previously. Thus it was suggested that TRH binds in an unprotonated, or neutral form, to its receptor and does not involve ionic interactions.

The TRH-R activates the PI pathway by coupling to the G-protein(s) G_q/G_{11} (Hsieh & Martin, 1992) which stimulate the enzyme phospholipase C (PLC). In certain systems, TRH has also been shown to

stimulate the adenylate cyclase pathway via G_s (Paulssen et al., 1992). Important regions in coupling of GPRs to G-proteins include the membrane proximal regions of CL3 and the membrane proximal region of the COOH tail (Dohlman et al., 1991; Strosberg et al., 1991; Probst et al., 1992). These regions are proposed to form amphipathic helical secondary structures (Segrest et al., 1990) which may directly contact and activate G-proteins. The amino acid variation found at the COOH tail of the mouse, rat and human TRH-Rs could be involved in TRH-R coupling to different second messenger pathways as was found for the prostaglandin PGE_2 EP3 receptor subtypes (Namba et al., 1993).

Other conserved GPR features found in the TRH-R include the D(E)RY sequence in the membrane proximal region of CL2 and conserved prolines in TM5, TM6 and TM7 (Fig. 4.3). Pro residues are believed to introduce 'kinks' or bends into the transmembrane domains for correct formation of the ligand binding pocket (Hibert et al., 1993).

Continued exposure of receptors to agonist can eventually result in a reduced response, known as desensitisation. Phosphorylation of receptors at Ser/Thr residues may contribute to receptor downregulation and, therefore, desensitisation (discussed in Chpt. 2.6.25-29). The TRH-R, coupled to the PLC pathway, does desensitise and does have potential phosphorylation sites (Fig.4.2), but work by Perlman and Gershengorn (1991) suggested that protein kinase C (PKC), a kinase activated by diacylglycerol (DAG), as part of the PLC second messenger pathway stimulated by TRH, does not appear to be involved. Stimulation of receptors by TRH also results in elevated $[Ca^{2+}]_i$, which might activate a Ca^{2+}/calmodulin-dependent protein kinase. Elevated calcium, however, does not seem to be responsible for desensitisation either, at least in GH3 cells (Perlman & Gershengorn, 1991). Other PKC isozymes in other cell types may produce different results.

The human TRH-R has 4 more Thr residues than the mouse TRH-R and the rat TRH-Rs have 3 more Thr residues than the mouse in the central region of CL3 (Fig. 4.4). These amino acid differences may reflect variation in phosphorylation of TRH-Rs between thyrotrophs and lactotrophs, or differences in G-protein-coupling between TRH-Rs in different cell-types.

The variant COOH tails might also be involved in differential TRH-R regulation by phosphorylation. The human and rat (412/411) TRH-Rs encode three extra Ser residues than the mouse TRH-R, and the long form of the rat TRH-R has two extra Thr residues than the human receptor at the COOH tail. Interestingly, the rat TRH-R_{387} has lost six serines and five threonines due to the 52bp deletion and frame-shift. Both the long and short forms of the rat TRH-R have been shown to co-exist in GH3 cells (de la Peña et al., 1992b) and Paulssen et al. (1992) have demonstrated activation of both the PI and adenylate cyclase pathways by the TRH-R in GH3 cells. Thus the rat TRH-R COOH tail differences might allow differential receptor regulation, or activation of multi-functional signalling pathways by TRH via different receptors within the same cell (Milligan, 1993) as was found for the prostaglandin EP3 receptors (Namba et al., 1993).

Internalisation of receptors is thought to occur following ligand-binding and activation of the second messenger pathway (Chpt. 2.6.26). No consensus signalling sequences for this process have yet been found, although the region encoded by the COOH tail has been implicated (Chpt 2.6.18-20). Two distinct domains that affect agonist-induced receptor internalisation have been characterised in the mouse TRH-R (Nussenzveig et al., 1993a). These include a domain between residues 335 and 368, and the need for a proximal Cys residue(s), either Cys^{335} or Cys^{337}. The Cys^{337} may be palmitoylated, so perhaps post-translational modifications are involved in internalisation. Nussenzveig et al. (1993b) have also reported that coupling to G-protein and PLC increases TRH-R internalisation. This supports the theory that a quaternary complex of ligand-receptor-G-protein-effector is internalised, unlike βARs, which are thought to dissociate from their adenylate cyclase-coupled G-proteins before internalisation (Lefkowitz & Hausdorff, 1991; Yu et al., 1993). It will be interesting to define the domains involved in desensitisation and internalisation of the human TRH-R, with respect to the COOH tail variation.

Chromosomal localisation of the human TRH-R gene

The localisation of the human TRH-R to the long arm of chromosome 8 (8q23) will prove useful if chromosomal abnormalities such as

translocations or deletions are discovered in this region of the genome. A search of the 10th edition of McKusick (1992) has so far revealed no chromosome alterations at 8q23 relevant to the TRH-R. An autosomal recessive phenotype, termed 'TRH deficiency: hypothalamic hypothyroidism', may possibly be caused by a TRH-R genetic defect, although this is only speculation (Foresti & Ferrari, 1985). Genetic defects at the level of point mutations as well as larger chromosomal abnormalities can also cause serious disease (Chpt. 2.6.30), and knowledge of the human TRH-R sequence will be helpful in determining whether the TRH-R is implicated in any clinical pathologies.

4.5 Summary

In conclusion, this chapter has reported the cloning of a cDNA encoding the human pituitary TRH-R protein with a predicted sequence of 398 amino acid residues. Comparison of the amino acid sequence of the human TRH-R with the previously published rodent TRH-Rs showed that all the TRH-R subtypes are highly homologous, except that each subtype encodes variant amino acids at the 3' end of the COOH . The human TRH-R belongs to the family of 7 transmembrane domain, G-protein-coupled receptors and it is believed that the COOH terminal region of this family of receptors may play an important role in receptor downregulation/internalisation, and possibly G-protein coupling. COS-1 cells expressing the human TRH-R showed high affinity receptor binding. Stimulation of these cells with TRH produced a typical phosphinositide response and mobilisation of intracellular calcium. The human pituitary TRH-R appears to be identical to a sequence obtained from human brain (Matre et al., 1993), but there may be multiple subtypes of the human TRH-R as yet undetected in brain and pituitary, as has been found for the rat TRH-R (de la Peña et al., 1992b). These possibilities are discussed further in Chpt. 6.

5 Tissue localisation studies of TRH-R messenger RNA in human

5.1 Introduction

The initial characterisation of the TRH-R utilised receptor radio-ligand binding studies using membrane homogenates from various mammalian species, and high affinity TRH analogues such as [^{3}H](3-Me-His^2)TRH. High affinity binding sites have been characterised in the anterior pituitary (Wilber & Seibel, 1973; Labrie et al., 1972; Gautvik & Lystad, 1981) and are widely distributed throughout the brain (Burt & Snyder, 1975; Burt & Taylor, 1980; Ogawa et al., 1981; Pazos et al., 1985) using these methods. By 1984, Johnson and colleagues (Johnson et al., 1984) had succeeded in solubilising TRH-Rs from rat brain, and their results suggested the existence of high and low affinity binding sites related to a single macromolecular complex. TRH-Rs have also been described in medullary neurons projecting to the spinal cord where they have been proposed to coexist with substance P and serotonin (Ogawa et al., 1985; Johansson et al., 1981).

Recent advances in molecular technology have resulted in the cloning of several TRH-Rs from mouse pituitary (Straub et al., 1990), rat pituitary (Zhao et al., 1992; de la Peña et al., 1992a,b; Sellar et al., 1993), human pituitary (Duthie et al., 1993a; Yamada et al., 1993) and human brain (Matre et al., 1993). The use of these TRH-R sequences as hybridisation probes has enabled techniques such as Northern blot analysis and *in situ* hybridisation (Whitman, 1991) to be used in studying the distribution of the TRH-R in different tissues. These methods are capable of determining receptor gene expression by hybridising DNA or RNA probes to the receptor messenger RNA (mRNA) as it is expressed in a particular tissue. A detailed study by Zabavnik et al. (1993) demonstrated the distribution of the TRH-R expression in rat pituitary, and in various regions of the rat brain, by *in situ* hybridisation.

TRH and TRH-like peptides have been localised to several tissues outside the CNS, including the retina (Schaeffer et al., 1977), gastro-intestinal system (Morley et al., 1977; Martino et al., 1978; Morley et al., 1979; Kawano et al., 1983), placenta (Shambaugh et al., 1977), and the

male reproductive system (Pekary et al., 1980; Feng et al., 1993a), prompting the search for TRH binding sites in these tissues. Localisation of TRH-Rs to particular tissues will help to determine possibles roles for TRH other than as a pituitary releasing hormone. TRH in the retina appears to be regulated by environmental lighting (Schaeffer et al., 1977) and this, together with the identification of TRH-R mRNA in the rat retina (Satoh et al., 1993), suggests some function for TRH in this tissue. A biological role for TRH in the male reproductive tract also appears likely, as TRH (Pekary et al., 1980; Feng et al., 1993a) and TRH-Rs have been co-localised to the rat testis by Northern analysis, membrane-binding studies and *in situ* hybridisation (Feng et al., 1993b). It is speculated that TRH may be an autocrine regulator of testosterone production and a paracrine regulator of spermatogenesis (Feng et al., 1993b).

TRH and TRH-like peptides have also been localised to the prostate (Pekary et al., 1980; Pekary et al., 1989; Fuse et al., 1990; Del RioGarcia & Smyth, 1990; Bilek et al., 1992; Gkonos et al., 1993). This raises the question of whether TRH binding sites are also expressed in the prostate. Having established the structure of the human TRH-R (Chpt 4) it now becomes possible to examine this question using molecular techniques such as Northern blotting and *in situ* hybridisation. Thus the aim of this study was to further the understanding of extra-pituitary functions for TRH by studying the distribution of TRH-R mRNA in various human tissues.

5.2 Materials and methods

Buffers and solutions used are described in Appendix I. All glassware was washed and baked before use to inhibit RNAse enzymes that destroy the RNA. Gloves were worn at all times.

5.2.1 Slide and tissue preparation for *in situ* hybridisation

The distribution of the human TRH-R mRNA in human pituitary, prostate, ovary and placenta was studied using *in situ* hybridisation (rat pituitary was used as a positive control). A normal human pituitary, obtained following post-mortem, was donated by Dr J Bevan, Aberdeen Royal Infirmary. The pituitary had been frozen at -70°C, eleven hours

after death. Ovarian tissue (in early follicular phase with a 6mm follicle, day 4 of the menstrual cycle) was obtained at surgery by Dr P Illingworth. Placental tissue was obtained by Dr S Cooper, immediately following routine delivery, from the Edinburgh Royal Infirmary. Prostatic tumour tissue (benign and malignant) was kindly donated by Dr J Waxman of the Hammersmith Hospital, London. The ovary, placenta and prostatic tissue was frozen immediately and stored in liquid nitrogen until use.

Frozen tissue was sectioned (10μm) in a Frigocut cryostat and thaw-mounted on to microscope slides (previously washed and baked) coated with 2% aminopropyltriethoxy-silane (APES). Sections were stored at -70°C prior to hybridisation.

5.2.2 Riboprobe preparation

The human TRH-R cDNA clone was amplified by PCR to produce a 410bp fragment of DNA encoding nucleotides 938 to 1347 of the TRH-R coding region. Transcripts of between 200bp and 800bp are thought to be optimal for tissue penetration (Whitman, 1991). Larger probes have to be hydrolysed, which is difficult to control and results in the loss of much of the probe's activity. The PCR product was purified as described in Chpt. 3.9.1 and subcloned into the TA cloning vector pCR™II (Chpt. 3.4). At least 1μg of this recombinant plasmid was linearised with the restriction enzymes Not1 and Sac1, which cut the vector on either side of the inserted PCR fragment. The linearised plasmid was extracted once with phenol/chloroform, and once with chloroform/IAA (24:1) and precipitated for an hour at -20°C. The plasmid was pelleted by microfuging at 13,000rpm for 10min. at 4°C, washed in 70% ethanol, air-dried and resuspended in 9μl double-distilled water. A sample (1μl) of the plasmid was electrophoresed on an agarose gel to determine the extent of linearisation.

The linearised plasmid was *in vitro* transcribed from either the SP6 promotor (Not1-cut plasmid) or the T7 promotor (Sac1-cut plasmid) with SP6 and T7 polymerases (Promega) respectively. The antisense strand was transcribed from the T7 promotor and represented the experiment, while the sense strand was transcribed from the SP6 promotor and represented the control. The transcription reaction mix contained 5XTSC buffer (Promega), 1μg DNA template, 10mM each of

rATP, rGTP and rCTP and 0.2mM rUTP, 50μCi [^{33}P]rUTP (Amersham), 20U RNA polymerase, 40U RNAse blocker (RNAsin, Promega) and double-distilled water to a total volume of 25μl. After 45min. incubation at 37°C, a further 1μl of the appropriate RNA polymerase was added to ensure a full-length transcript was obtained, and transcription continued for a further 45min. Following transcription, 2μl tRNA (10mg/ml) was added as a carrier molecule and the DNA template was degraded with DNAse I (10U, Boehringer) for 10min. at 37°C. The riboprobes were extracted once with phenol/chloroform and once with chloroform/IAA (24:1) and precipitated for at least an hour at -20°C. The riboprobes were pelleted by microfuging at 13,000rpm for 15min. at 4°C, washed with 70% ethanol, centrifuged, air-dried and resuspended in 20μl double-distilled water. Incorporation of ^{33}P was determined by adding 1μl of riboprobe to 1ml scintillation fluid and counting on a Rackbeta counter. Riboprobes were stored at -20°C until use.

4.2.3 *In situ* hybridisation

The method used was adapted from that described previously (Peters et al., 1991). Frozen tissue sections were dried rapidly with a hair-dryer and fixed in fresh 4% paraformaldehyde (5min.). Fixed sections were washed twice in 0.1M phosphate buffer (5min. each) and once in double-distilled water with shaking. Sections were washed in TEA buffer for 2min. and treated with TEA buffer (containing 0.25% acetic anhydride) for 10min. with shaking to reduce non-specific binding of the negatively charged probe to the positively-charged APES used to coat the slides. The melting temperature (Tm) for hybridisation was determined using the following equation Tm = 81.5 + 16.6(log[M]) + 0.41(%G+C) - 0.65(F) - 820/l, where M = salt concentration of buffer, G+C = %G:C content of the riboprobe, F = formamide concentration of buffer and l = length of the riboprobe (Chan et al., 1989). The sections were rinsed in 2XSSC, blotted and prehybridised with a few drops of prehybridisation buffer at 42°C (Tm -15°C to -25°C) for an hour in a Hybaid oven. Prehybridisation was performed to reduced non-specific binding of the riboprobe.

During the prehybridisation step, the sense (SP6) and antisense (T7) riboprobes were diluted in hybridisation buffer ($1x10^6$ counts in 40μl hybridsation buffer for each slide), heated to 60°C for 3min. and placed

directly on ice until required. Following the prehybridisation step, slides were blotted and placed in sterile dishes. Diluted riboprobe (40µl) was added to each section and covered with FMC Gelbond film (hydrophilic side up). Vials of sterile water were also placed in the dishes to prevent the sections from drying out. Tissue sections were incubated at 42°C (Tm -15°C to -25°C) overnight.

5.2.4 Washing of hybridised tissue sections

Following hybridisation, the sections were washed in 4XSSC to remove the coverslips. Tissue sections were then incubated with prewarmed RNAse buffer containing 20µg/ml RNAse A at 37°C for 30min. to remove non-specific riboprobe, and rinsed once with RNAse A buffer for a further 30min. Washing was performed at low stringency in 2XSSC for 30min. at room temperature, with shaking, followed by 1XSSC and then 0.5XSSC both for 30min. at room temperature. The tissue sections were dehydrated through an ascending series of industrial methylated spirit [IMS], (50%, 85%, 100%) containing 300mM ammonium acetate for 3min. each, and air-dried.

5.2.5 Autoradiography of hybridised tissue sections

Slides with washed and dried hybridised tissue sections were prewarmed on a hot-plate at 45°C. Coating of slides with prewarmed (45°C) Kodak NTB2 autoradiography emulsion was performed in a dark-room. Once coated, the slides were placed in a rack in a sealed plastic box lined with damp tissue and left at room temperature for 4 hours in a light-proof container. The slides were transferred to black polyacetyl boxes (Raymond A. Lamb) containing a perforated vial of silica gel and double-wrapped in tinfoil. Sections were exposed to the emulsion at 4°C for 10 to 21 days.

Before developing, slides were prewarmed to ~15°C. The developer (Kodak), stop solution (water), and fixative (Kodak UNIFIX, diluted 1/5) were cooled to ~15°C on ice before use. In the darkroom the slides were processed through each solution as follows: developer (4min.), stop solution (20 seconds), fixative (5 to 10min.) and washed in distilled water for 10 to 15min. The slides were rinsed in running water for 15min. before staining with haemotoxylin (stains nuclei purple) for 1 to 5min.

Excess haemotoxylin was removed with 1% (v/v) acid alcohol for 20 seconds and the slides placed in Scott's tap water. After each of these treatments, the slides were rinsed in fresh, running tap water. The tissue sections were examined to determine the extent of staining, and the process repeated if necessary.

The counterstained sections were dehydrated through an ascending series of ethanol (70%, 80%, 100%) and placed in Histoclear (National Diagnostics) for 5min. before transferring to Xylene (BDH) in a fume-hood. Sections were mounted in Eukitt (O. Kindler GmBH & Co.) under a coverslip and viewed under dark-field microscopy to determine the localisation of the radioactive probe, revealed by the distribution of silver grains in the photographic emulsion.

5.2.6 RNA extraction

Total RNA was extracted from 293 human embryonic kidney fibroblast cells stably expressing a 3.5kb rat TRH-R cDNA clone (293-E2 cell line, established by Jelka Zabavnik), as a positive control, and from untransfected 293 cells (negative control). Prostate mRNA (poly A^+) was obtained from Clontech. The method used was essentially that of Chomczynski & Sacchi (1987). Cultured monolayers of 293 cells were placed on ice, the culture media removed and the cells washed with ice-cold phosphate buffered saline (PBS). Solution D (1ml to 2ml, Appendix I) was added to the monolayer to lyse the cells, and the viscous solution removed to polypropylene tubes. The dish was washed with a further 1ml to 2ml (depending on the size of the culture) of solution D and pooled with the previous extract.

After cell lysis, the following solutions were added sequentially (per 1ml solution D), with shaking after each addition; 0.1ml 2M sodium acetate (pH 4.0), 1ml water-saturated phenol and 0.2ml chloroform/isoamyl alcohol (49:1). The final mixture was shaken vigorously for 10 seconds and cooled on ice for 15min. Samples were centrifuged for 20min. at 10,000g and 4°C in a pre-cooled Beckman fixed-angle rotor (JA 20.1). The aqueous phase, containing the RNA, was carefully removed to a sterile tube, ensuring no contamination from proteins at the interphase, and mixed with an equal volume of cold isopropanol. The RNA was left to precipitate at -20°C for at least an hour,

and centrifuged as described above. The supernatant was discarded, and the translucent RNA pellet redissolved in 0.3ml to 1ml solution D. The solution was transferred to a sterile 1.5ml Eppendorf tube before adding an equal volume of cold isopropanol, inverting to mix, and precipitating at -20°C for at least an hour. RNA was pelleted by microcentrifuging at 13,000rpm for 10min. at 4°C. The supernatant was discarded carefully, and the pellet resuspended in cold 75% ethanol, microcentrifuged for 2min. and the supernatant again discarded. At this point, the pellet was either freeze-dried and resuspended in 50µl to 200µl of sterile water by heating at 65°C for 10min., or it was resuspended in fresh 75% ethanol. Both were stored at -70°C.

To determine the concentration and purity of the extracted RNA, a sample was thawed gently on ice, and then diluted (10^{-2}) in double-distilled water. The absorbance of the sample was determined at an optical density (OD) of 260nm and 280nm. The ratio of the readings (260/280) determined the purity of the sample - a ratio of 2.0 denoted pure RNA. An OD_{260} of 1.0 = 40µg/ml RNA.

5.2.7 Separation of RNA using formaldehyde gels

Total RNA (20µg) extracted from 293-E2 cells (expressing the rat TRH-R) and 293 cells (untransfected), 2µg polyA$^+$ prostate mRNA (Clontech) and 3µg RNA markers (Promega) were electrophoresed on 1.5% formaldehyde gels. Molecular biology grade agarose (1.5g, SeaKem) was dissolved in 85ml double-distilled water in a baked conical flask. The gel was cooled to ~60°C and placed in a fume-hood where 5.4ml formaldehyde (37% solution) was added and the gel poured immediately, leaving to set for 30min. The required volume of RNA was placed in a sterile Eppendorf tube, the volume adjusted to 4.4µl with sterile water and 15.6µl sample buffer (Appendix I) added. Samples were heated at 60°C for 5min. and 8µl dye solution (Appendix I) added, mixed well and the RNA samples loaded on to the prepared gel. Gels were run overnight at 35V in 1X MOPS/EDTA running buffer (Appendix I) and the RNA visualised and photographed under u/v light.

5.2.8 Northern blot analysis of RNA

RNA was transferred to Hybond N nylon membrane (Amersham) overnight, using 20XSSC running buffer (Appendix I). The apparatus was set up as described for Southern blotting in Chpt. 3.7.4. Following transfer, the RNA was fixed to the membrane by u/v cross-linking, as described in Chpt. 3.2.2 and was stored in a sealed bag at 4°C until use. RNA gels were also photographed under u/v light after blotting to determine the efficiency of transfer. The RNA was hybridised to a 581bp human TRH-R cDNA PCR product (nucleotides 227 to 808), random-primer labelled with ^{32}P dCTP (Chpt. 3.8.3).

A multiple tissue Northern (MTN) blot (Clontech) was hybridised with the same probe, according to manufacturer's instructions. This Northern blot was obtained commercially owing to the difficulty in obtaining certain human tissue for RNA extraction. The blot contained 2μg of pure poly A^+ mRNA in each lane, from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The blot was washed at low stringency (2XSSC, 0.1% SDS at room temperature for several washes, and then at 50°C with 1XSSC, 0.1%SDS) to allow for variation in TRH-R transcripts in different tissues.

5.3 Results

5.3.1 Localisation of TRH-R mRNA by *in situ* hybridisation

The human pituitary tissue available was poorly preserved and the RNA appeared to have degraded, necessitating the use of rat pituitary as a positive control. Positive hybridisation to the antisense human TRH-R riboprobe was obtained in the anterior and neurointermediate lobes of rat pituitary, but not the posterior lobe (Fig. 5.1A). The negative controls (sense probe) had high background, but the signal appeared to be evenly distributed over the whole tissue, with no specific hybridisation (Fig. 5.1B). The high background was attributed to labelling the riboprobes with ^{33}P (a high energy radio-isotope) and also to the long exposure time used (19 days). Slides developed after only one week had lower backgound levels (data not shown). Neither the ovary nor the placenta gave clear results.

The results presented for the prostate tumour tissue were obtained following several trial runs with a small sample number (n=2) to establish optimal conditions. Results from 9 benign and 8 malignant tumours are presented in Table 5.1. Strong hybridisation to the antisense probe (T7) was visualised in the glandular epithelium (GE) of 3/8 benign tumours, 5/8 also showed positive hybridisation to stromal tissue. In one benign tumour (patient 6) no clear hybridisation above background levels was determined (ND).

The malignant tumour tissue gave similar results, with 3/6 showing strong hybridisation to glandular epithelium, 3/6 showing strong stromal signal and 2 not determined (ND). There were no obvious differences observed between hybridisation signals for the two types of tissue. The sense (SP6) riboprobe seemed to 'shadow' the antisense probe in some tumours (Fig. 5.2B), but at a much lower level of hybridisation. This shadowing is probably an artifact as it did not occur when the slides were developed after only 7 days incubation (data not shown). At this stage, positive hybridisation to the antisense probe was very faint but clearly visible. Figs. 5.2 to 5.4 show some of the results obtained with the human TRH-R riboprobe hybridising to prostatic tumour tissue.

5.3.2 Northern blot analysis of the TRH-R

The human prostate polyA$^+$ mRNA showed a clear hybridisation signal at approximately 4.7kb with a second transcript at approximately 2.7kb, and possibly a third at 1.6kb (Fig. 5.5). Washing the blot more stringently revealed the smaller transcripts more clearly (data not shown). The human TRH-R probe hybridised to a transcript of ~6.4kb in 293-E2 cells, with a second, less abundant transcript at ~4.9kb. No hybridisation signal was visible in the RNA from untransfected 293 cells (negative control).

The Northern blot obtained from Clontech (Fig. 5.6) proved difficult to analyse. Multiple transcripts were observed in all tissues (Table 5.2), except the brain which had no clear hybridisation signals. The TRH-R does exist in the brain, however, as it has recently been isolated from a human brain cDNA library (Matre et al., 1993). Liver gave a very strong signal which could not be resolved at low stringency.

Tissue type	Patient	Probe	Hybridisation signal	Comments
Rat pituitary	----	T7	+++++	AP and NIL only
		SP6	++	
Human pituitary (male)	----	T7	+	ND
		SP6	+	
Human ovary	----	T7	+++	ND
		SP6	+++	
Human placenta	----	T7	+++	ND
		SP6	+++	
Benign prostatic hyperplasia (BPH)	1	T7	+++++	GE only
		SP6	++	
	2	T7	+++++	GE only
		SP6	++	Slight 'shadowing'
	3	T7	+++++	Stroma
		SP6	+	
	4	T7	++++	GE and stroma
		SP6	++	Slight 'shadowing'
	5	T7	++++	'Edge effect' in stroma
		SP6	+++	Strong shadowing
	6	T7	++	ND
		SP6	++	
	7	T7	++++	Signal in some GE
		SP6	+++	Shadowing, poor tissue preservation
	8	T7	++++	Stroma
		SP6	++	
	9	T7	++++	GE & stroma
		SP6	+++	
Prostatic Carcinoma (Malignant)	10	T7	++++	Stroma
		SP6	+	
	11	T7	+++++	Stroma
		SP6	++++	
	12	T7	+++++	GE
		SP6	+	
	13	T7	+++++	GE, some stroma
		SP6	+	
	14	T7	+++++	Stroma
		SP6	+	
	15	T7	++	ND
		SP6	+	
	16	T7	+++	ND
		SP6	+++	
	17	T7	+++++	Some GE
		SP6	+	

Table 5.1

In situ hybridisation results obtained with human TRH-R riboprobe. Anterior pituitary (AP), neurointermediate lobe (NIL). Antisense (T7), sense (SP6); strong hybridisation signal (+++++), weak signal (+); Glandular epithelium (GE); Not determined (ND).

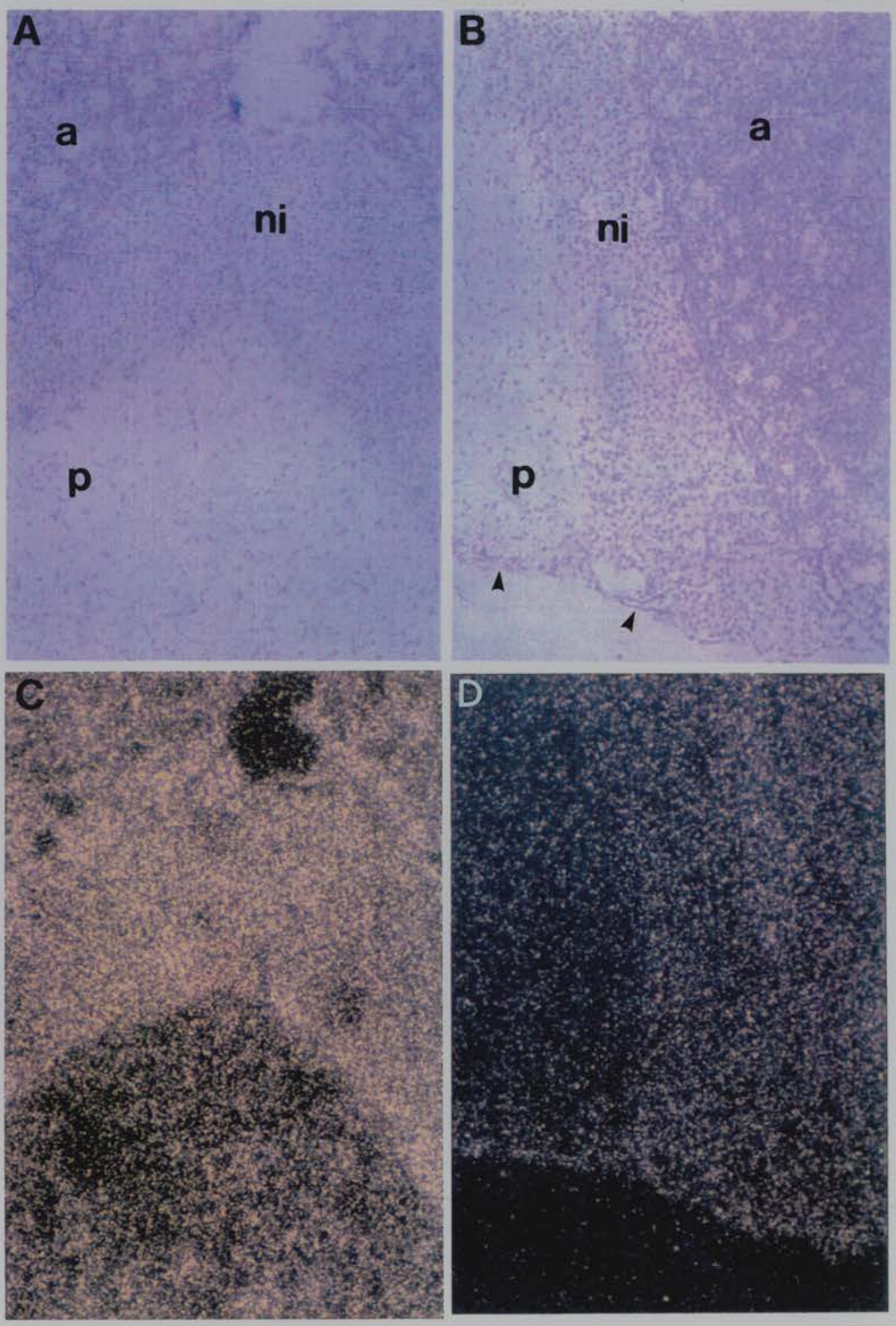


Fig. 5.1

Photomicrographs of rat pituitary gland. Light-field photomicrographs (A) and (B) illustrating the anterior lobe (a), neurointermediate lobe (ni) and posterior lobe (p). The sections were hybridised with the ^{33}P-labelled antisense human TRH-R riboprobe (C) or sense riboprobe (D). No expression was found in D. Arrows indicate edge of tissue.

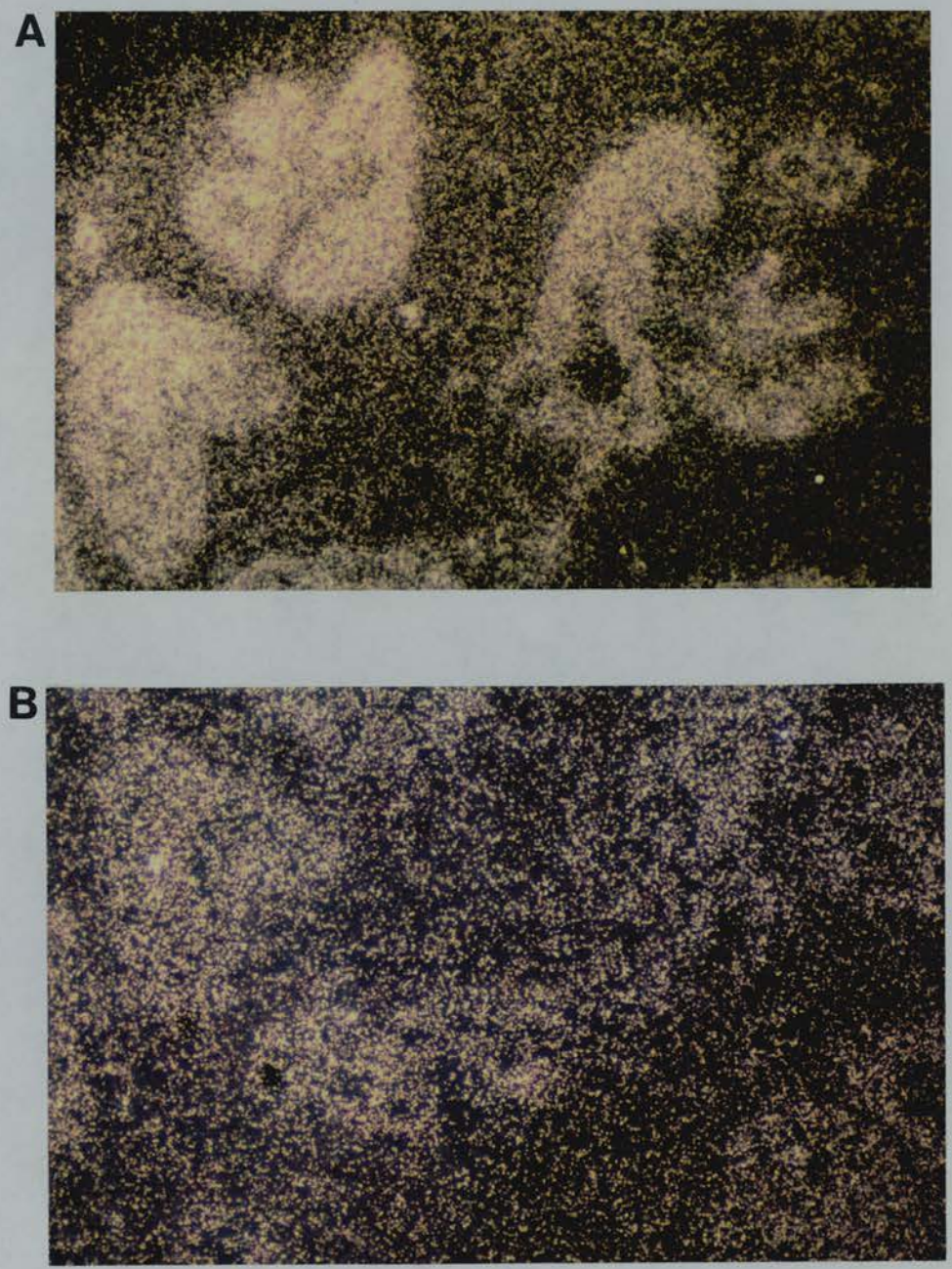


Fig. 5.2

Photomicrographs (X10 objective) illustrating human prostate tumour tissue (benign) - Patient 7, hybridised to the antisense (A) or sense (B) ^{33}P-labelled human TRH-R riboprobe. The sense probe (B) shows slight 'shadowing' of the antisense probe (A).

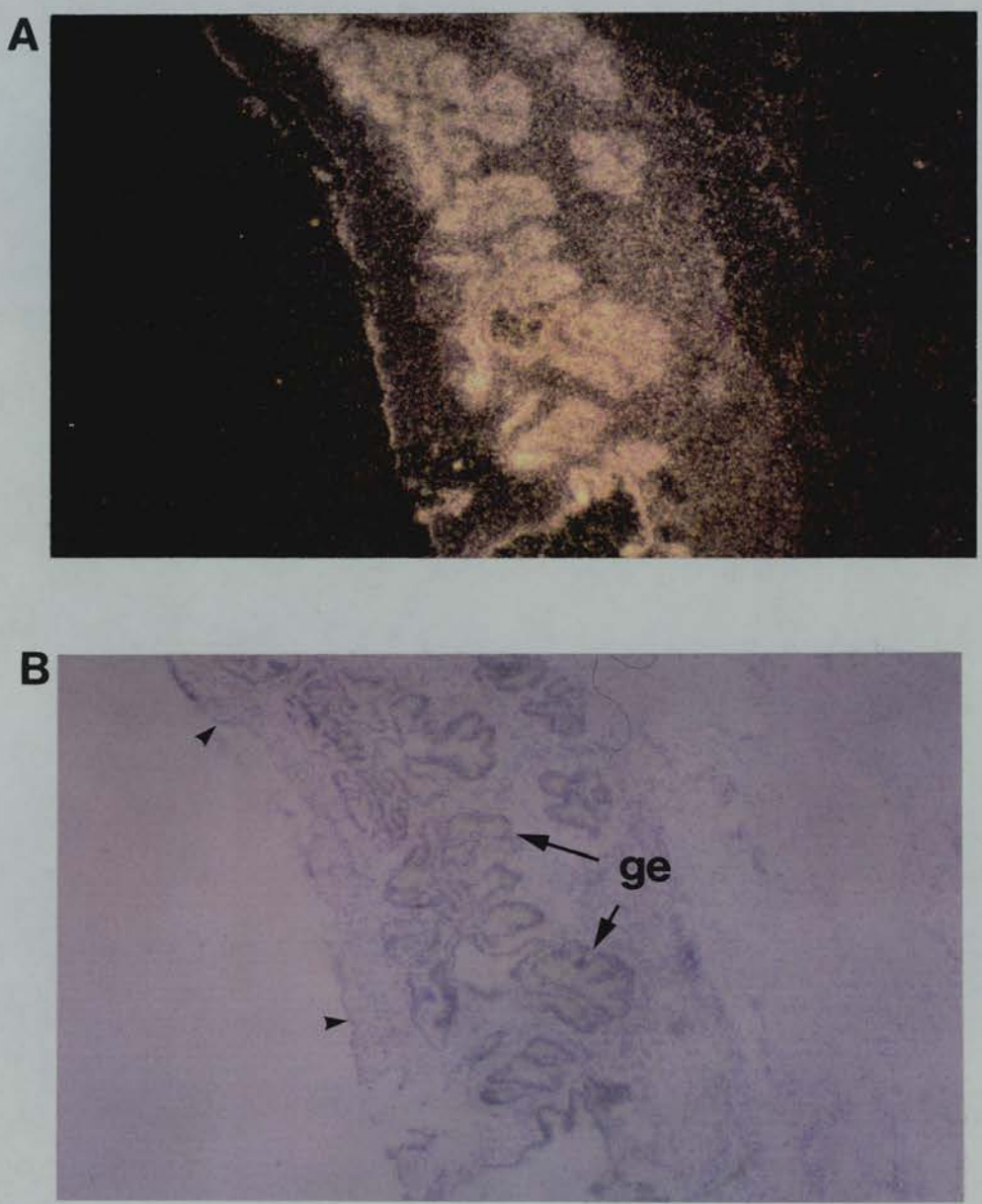


Fig. 5.3

Photomicrographs (X4 objective) illustrating human prostate tumour tissue (benign) - Patient 4, hybridised to the antisense (A) ^{33}P-labelled human TRH-R riboprobe. (B) light-field photomicrograph illustrating glandular epithelium (ge). Arrows indicate edge of tissue.

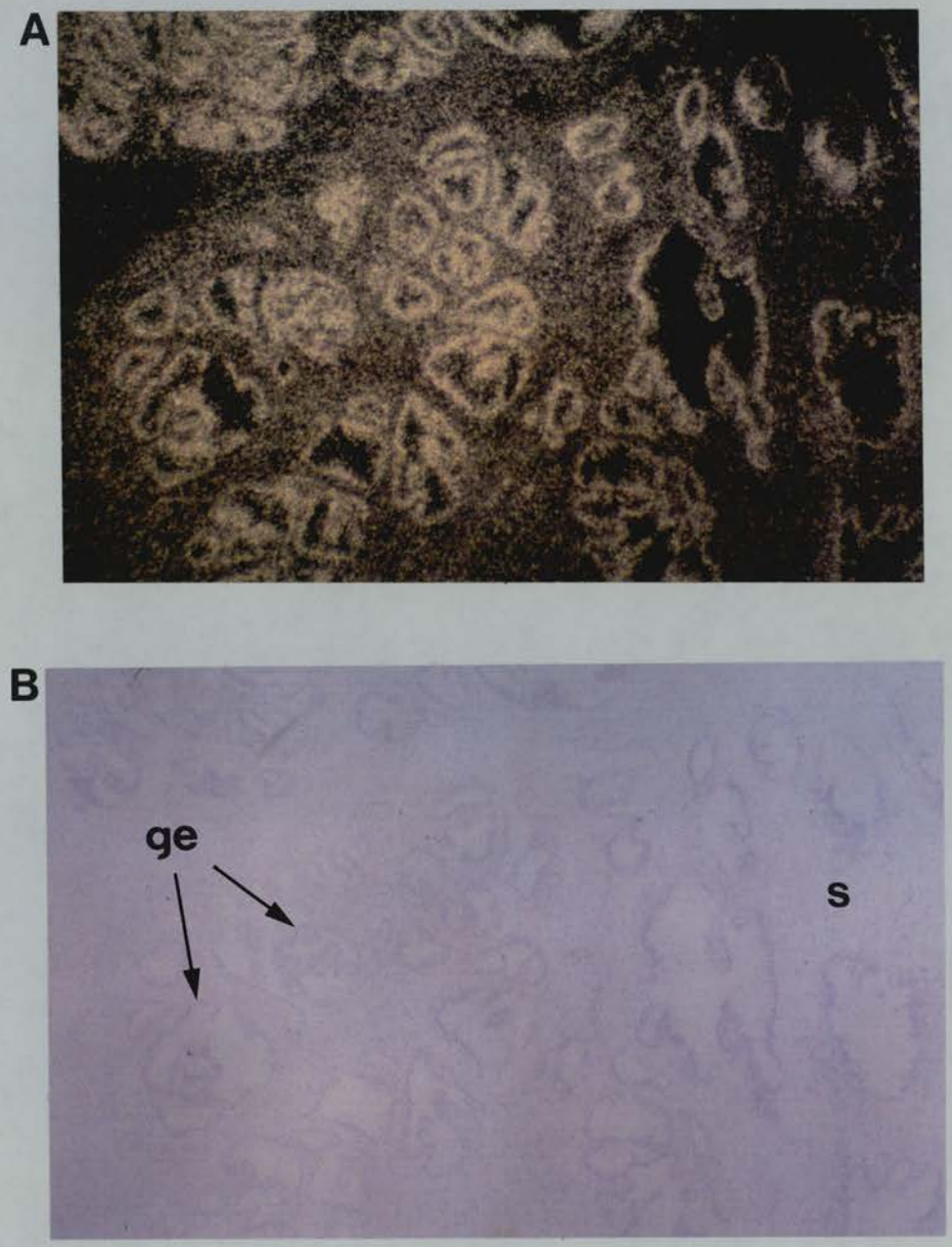


Fig. 5.4

Photomicrographs (X10 objective) illustrating human prostate tumour tissue (malignant) - Patient 12, hybridised to the antisense (A) ^{33}P-labelled human TRH-R riboprobe. (B) light-field photomicrograph illustrating glandular epithelium (ge) and surrounding stroma (s).

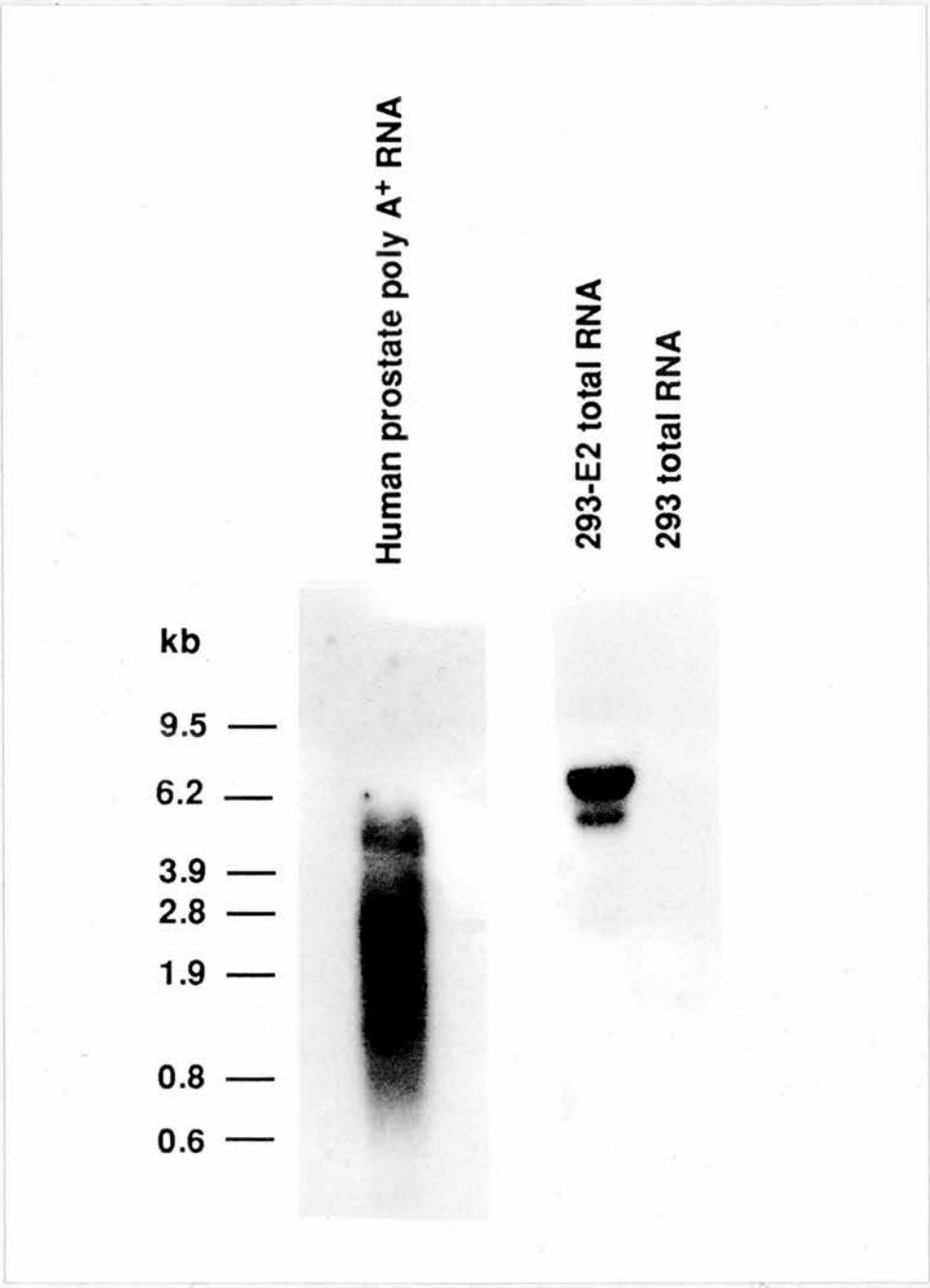


Fig. 5.5

Northern blot analysis of poly A^+ mRNA from human prostate and total RNA from 293 cells hybridised to a human TRH-R PCR fragment. Poly A^+ mRNA (2μg) from human prostate (Clontech) and total RNA (20μg) extracted from 293-E2 cells transfected with the rat 3.5kb TRH-R cDNA, and from untransfected 293 cells was subjected to Northern blot analysis and hybridised to a 581bp human TRH-R PCR fragment probe as described in section 5.2.7-8.

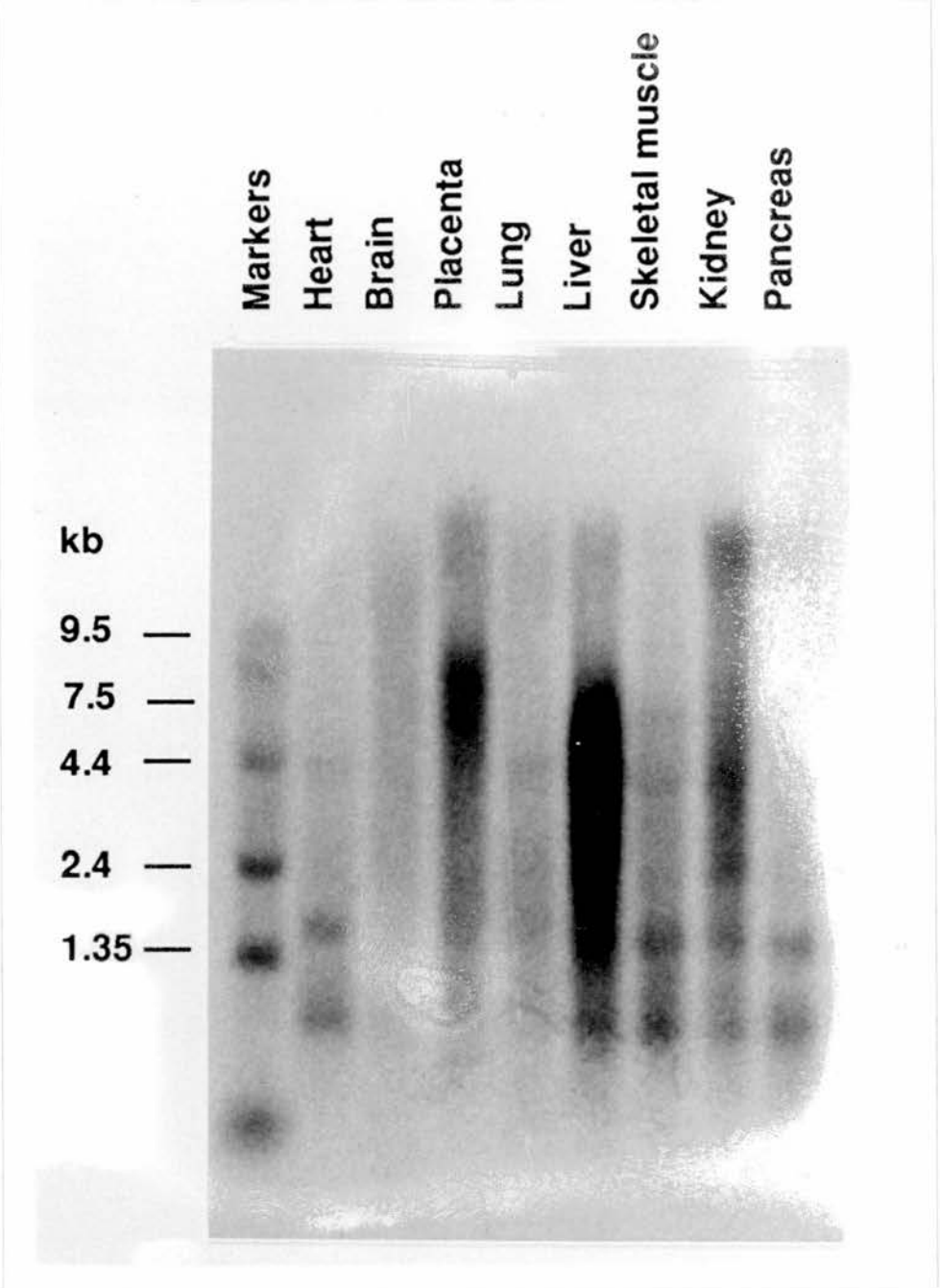


Fig. 5.6

Northern blot analysis of poly A^+ mRNA from a range of human tissues. The multiple tissue blot (Clontech) contained 2μg poly A^+ mRNA in each lane and was hybridised to a 581bp human TRH-R PCR fragment probe as described in section 5.2.7-8.

Tissue	**~Trancript sizes (kb)**					
293-E2 cells (rat TRH-R)	-	6.2	4.9	-	-	-
Prostate	-	-	4.7	2.7	1.6	-
Heart	-	-	4.4	-	1.6	0.8
Brain	-	-	-	-	(1.6)	-
Placenta	(8.0)	-	4.4	-	1.6	0.8
Lung	-	-	4.4	-	(1.6)	-
Liver	(7.5)	-	(4.4)	(2.4)	1.6	0.8
Skeletal muscle	7.5	5.9	4.2	2.4	1.6	0.8
Kidney	-	-	4.2	2.4	1.6	0.8
Pancreas	-	-	-	-	1.6	0.8

Table 5.2

RNA transcript sizes obtained from Northern analysis of the multiple human tissue in human tissue Northern blot (Clontech) hybridised to the 581bp human TRH-R PCR fragment probe. Figures in brackets represent transcripts that were not clearly visible.

Washing at higher stringency suggested that the signal consisted of two transcripts of ~7.5kb and 4.4kb, although the results were still not very clear (data not shown). The low molecular weight transcripts appeared to be the most abundant, while transcripts of a size expected of the TRH-R (3.5kb in GH3 cells [de la Peña et al., 1992a; Zhao et al., 1992]; 3.8kb in rat testis [Feng et al., 1993b] and 3.8kb in rat retina [Satoh et al., 1993]) were barely visible. Skeletal muscle expressed 4, or possibly 5 transcripts ranging from ~7.5kb to 0.8kb, while the only the two smaller transcripts of 1.6 and 0.8kb were visible in the pancreas. No transcript corresponding to ~4kb was visible in the pancreas.

5.4 Discussion

In situ hybridisation analysis of extra-pituitary tissues using the human TRH-R probe provided some interesting results. Rat pituitary acted as a positive control in the absence of fresh human tissue, and gave a positive hybridisation signal to the human probe (Fig. 5.1). The fact that no clear results were obtained in placenta and ovary does not necessarily mean that the TRH-R is not expressed in these tissues, especially in the placenta, to which TRH itself has been localised (Shambaugh et al., 1977). Different receptor subtypes may be present that would not hybridise to this particular region of the TRH-R (nucleotides 938 to 1347), or receptor expression may be developmentally regulated. Northern blot analysis of prostate poly A^+ mRNA suggested the presence of TRH-R-like transcripts and is discussed below.

Putative TRH-Rs in the prostate

In situ hybridisation studies using the human TRH-R isolated as described in Chpt. 4, gave strong, positive results in human prostate tissue, both benign and malignant. In some sections, the signal was clearly localised to the glandular epithelium, whilst in others, positive hybridistion was found in the surrounding stromal tissue (Figs. 5.2 to 5.4). In certain tissue sections, some of the glandular epithelium showed positive hybridisation to the TRH-R probe, whilst some did not. The results revealed no obvious differences between benign and malignant tissue.

The prostate gland is an accessory organ of male reproduction and its growth and differentiation is regulated predominantly by androgen secretion, as well as by oestrogens, prolactin, insulin and possibly other

The prostate gland is an accessory organ of male reproduction and its growth and differentiation is regulated predominantly by androgen secretion, as well as by oestrogens, prolactin, insulin and possibly other growth factors (Meikle, 1989). The weight of the gland increases with age and the tissue often becomes hyperplastic in men at around age 45. This hyperplasia is related to androgen secretion as men castrated before puberty do not develop benign prostatic hyperplasia (BPH) (Meikle, 1989). The glandular epithelium is strongly influenced by stromal growth factors stimulated by androgens, and receptors for peptide hormones including prolactin (Aragona et al., 1977) and GnRH (Qayum, 1990; Waxman, 1991) have been reported in the prostate. BPH is successfully treated with surgery, although endocrine therapy that could act to prevent the disease would be highly desirable.

Cancer of the prostate is the second most common form of malignancy in adult males and more than 60% of cases occur in men over 70 years of age (Lippman, 1989). Both BPH and prostate carcinoma may respond to anti-androgens, but there are differences between the two diseases, including the site of origin within the gland. BPH occurs centrally, whereas carcinoma is usually peripheral (Lippman, 1989). GnRH agonist administration has been found to reduce tumour growth by creating a paradoxical decrease in LH and FSH from the pituitary and subsequent reduction in gonadal steroids (Waxman, 1991). Such agonists may also down-regulate GnRH-Rs in the prostate (Qayum et al., 1990). GnRH agonists avoid the psychological effects of castration, however, potentially non-reversible testicular damage has been reported as a result of this type of therapy (Decensi et al., 1993).

Levels of TRH/TRH-like peptides in the normal prostate decrease with age and their decreased expression in prostate cancer has also been reported (Gkonos et al., 1993). The discovery of putative prostatic TRH-Rs both by *in situ* hybridisation and Northern analysis reported in this chapter, implies a biological role for TRH in this tissue. Gordeladze at al. (1988) reported that TRH stimulated adenylate cyclase in both normal, hyperplastic and cancerous prostate, supporting the existence of TRH binding sites in this tissue. They also reported that TRH stimulation of adenylate cyclase was lower in hyperplastic tissue, and even lower in prostate carcinoma. It is tempting to speculate that the observed age-

related reduction in TRH levels in normal prostate, and in prostate carcinoma, may somehow be related to the onset of this disease.

Northern blot analysis

Multiple transcripts hybridising to the 581bp TRH-R probe appeared in all the human tissues analysed by Northern blotting, and were strongest in the liver and weakest in the brain. TRH-R binding has been reported in the liver (Burt & Snyder, 1975) and a TRH-R has recently been isolated from a brain cDNA library (Matre et al., 1993). The blot was washed at low stringency and thus the transcripts may represent other genes with some homology to the TRH-R. The transcripts could also represent alternatively spliced versions of the TRH-R gene. This possibility is discussed in Chpt. 6. Another prospect is that these are aberrant transcripts which are transcribed from the same gene, but are not translated into peptides, due to truncation, or extension of the mRNA at either the 5' or 3' terminus (Ivell, 1992). Several examples of this type of aberrant transcription have recently been reported for genes that are transcribed in both brain and testis, such as the pro-opiomelanocortin gene (Jeannotte et al., 1987; Lacaze-Masmonteil et al., 1987). Aberrant transcripts tend to occur in greater abundance than the normal-length functional transcripts, can appear in different cell types from the functional variants, and also show a high degree of species specificity (Ivell, 1992).

A faintly hybridising band of ~4kb was observed in heart, lung, skeletal muscle, kidney, prostate and possibly placenta and liver (Table 2.2). This may represent a low abundance, functional TRH-R transcript, since it roughly corresponds to the range of TRH-R transcript sizes from 3.5kb to 4kb observed in GH3 cells (de la Peña et al., 1992a; Zhao et al., 1992) and the rat testis (Feng et al., 1993). The LH-R gene (a member of the GPR family) is expressed as multiple transcripts of varying sizes in the testis, depending on the species (Loosfelt et al., 1989; Tsai-Morris et al., 1991; Wang et al., 1991). Some of these transcripts are larger than the open-reading-frame, and some are smaller, as was found for the human TRH-R (Table 2.2). Multiple transcripts were also found for the GnRH-R in sheep (Illing et al., 1993; Brooks et al., 1993).

Ivell (1992) suggested that non-functional transcripts may arise from 'leaky transcription'. In this case, particular combinations of transcriptional elements may initiate, or terminate transcription at sites other than the correct initiation/termination consensus sequences. This effect could also explain the unusually large transcripts observed in the 293-E2, positive control RNA. Although the transfected rat TRH-R cDNA was only 3.5kb, its insertion site into the genome of the 293 cell line might affect its termination signals and interactions with the transcription machinery of the cell.

These results provide interesting preliminary data and open up several possibilities for future work. The TRH-R appears to be highly expressed in the glandular epithelium and, to a lesser extent, in the stroma of human prostate tumour tissue (benign and malignant). Similar studies with animal models would be helpful in investigating the expression of the TRH-R in relation to the development of the animal and in relating the expression of the receptor to its peptide. It would also be interesting to clone and sequence the TRH-R from a human prostate cDNA library to confirm these findings and to compare the prostatic TRH-R with the pituitary and brain TRH-Rs, as no human TRH-R isoforms have yet been cloned. Ligand binding studies on prostatic tissue would also help to characterise the nature of these putative TRH binding sites. The results from the Northern blot analysis emphasise the importance of providing functional data for observed gene transcripts. Time constraints prevented further studies on this project.

5.5 Summary

This chapter reports the localisation of putative TRH-R mRNA transcripts to a variety of human tissues. Significant positive hybridisation to a 410bp human TRH-R riboprobe was observed in human prostate tumour tissue (benign and malignant) and was supported by Northern analysis of prostate poly A^+ mRNA, which revealed transcripts of ~4.7kb, 2.7kb and 1.6kb. Multiple transcripts were also observed in a variety of human tissues and may represent homologous genes, aberrant TRH-R transcripts or alternatively spliced versions of the gene (Ivell, 1992). Further work is needed to establish a biological role for TRH in the prostate.

6 Structural analysis of the mouse TRH receptor gene.

6.1 Introduction

Unlike prokaryotic genes, eukaryotic genes are very often encoded by discontinuous DNA segments or exons, separated from each other by intervening sequences known as introns. Introns generally do not form part of the mature mRNA and are removed by a precise excision, or splicing mechanism. Splice sites are recognised by consensus sequences at the 5' and 3' boundaries of the introns, and splicing is performed by the interaction of small nuclear RNA molecules (snRNAs) with these sequences. In most genes studied so far, each exon is incorporated into the mature mRNA transcript through the invariant ligation of pairs of consecutive splice junctions. In some cases, however, non-consecutive exons can be joined together by utilising alternative splice sites. This process of alternative splicing allows a particular gene to produce more than one protein from a single coding sequence, and the choice of splice sites may be developmentally regulated or cell-type specific (Breitbart et al., 1987).

Alternative splicing does occur in some G-protein-coupled receptor (GPR) genes and has recently been shown to produce receptor isoforms capable of activating different second messenger pathways (Chpt. 2.6.31). The discovery of TRH-R subtypes with variant COOH tails (Chpt. 4) has raised the question of alternative splicing in the TRH-R gene, particularly as the sequence variation in each subtype has amino acid 392 as a common point of sequence divergence. The mouse TRH-R_{393} isolated from the mouse TtT thyrotroph cell line (Straub et al., 1990) encodes only one residue after amino acid 392. Several rat TRH-R isoforms have been isolated, including a TRH-R_{412} (Zhao et al., 1992; de la Peña et al., 1992a) from rat anterior pituitary GH4C1/GH3 tumour cell lines and a rat TRH-R_{411}, isolated from a pituitary cDNA library (Sellar et al., 1993). These receptors encode 20 and 19 amino acids respectively, after the breakpoint at residue 392. TRH-R_{411} has a deletion of 3 nucleotides resulting in the loss of one amino acid, residue 393 (Sellar et al., 1993).

There are several other methods of generating protein diversity from a single gene. In some cases, intronic sequence is actually 'retained' in the mRNA, in-frame with the rest of the coding region, resulting in the translation of a longer protein. In this case, the splice sites exist within the coding region (Breitbart et al., 1987; Smith et al., 1989). Most interestingly, another rat receptor subtype, TRH-R_{387} (de la Peña et al., 1992b) has been cloned from GH3 cells and appears to undergo this form of alternative splicing. This receptor contains a 52bp deletion in the COOH terminus, which causes a frame-shift in the coding sequence and results in 12 variant residues at the COOH terminus. PCR analysis was used to demonstrate that the nucleotides encoding this stretch of sequence are not contained on a separate exon and can be spliced in (TRH-R_{412}) or out (TRH-R_{387}) of the mRNA to produce two receptor proteins from one gene. The 3' splice junction of this retained intron occurs at residue 392. It was also shown that both the long and short form of the rat TRH-R can be coexpressed in GH3 cells (de la Peña et al., 1992b).

The mechanisms by which specific exons can be spliced in and out of genes is not currently understood, although it has been proposed that the presence, or absence, of particular splicing factors in a cell directs the preferential use of a particular set of exons (Smith et al., 1989 - Review; Lees-Miller et al., 1990). This does not explain the ability of a cell to produce more than one form of the same gene at the same time, as was found for the long and short forms of the rat TRH-R (de la Peña et al., 1992b).

Yet another TRH-R subtype has been isolated from a human pituitary cDNA library (Duthie et al., 1993a - Chpt. 4, Yamada et al., 1993) and human brain cDNA library (Matre et al., 1993). The human pituitary and brain receptors are identical and share high sequence homology with the rat and mouse TRH-Rs except at the 3' end of the COOH tail. The human TRH-R also has residue 392 as a point of sequence divergence, encoding 6 variant amino acids after this point.

The observation that the COOH tails of the rodent TRH-R subtypes showed sequence variation at the same site (see Fig. 4.4 in Chpt. 4), suggested that an alternative splicing mechanism may be responsible, perhaps the variant COOH tails were encoded on separarate exons? The obvious step was to clone a TRH-R gene to answer this question. The

mouse gene was chosen rather than the rat or human genes since the long term aim of the project was to study the gene *in vivo* using transgenic animal models, and the mouse is generally the species of choice in transgenic experiments. This chapter reports the cloning and characterisation of a mouse TRH-R gene, and focuses on the region of interest at the COOH tail.

6.2 Materials and methods

As for Chpt. 4, the general methods for isolating genes from commercially prepared libraries has been described in Chpt. 3. This section deals, therefore, only with specific information relevant to the cloning and characterisation of the mouse TRH-R gene.

6.2.1 Screening an EMBL3 mouse genomic library

Mouse genomic DNA, (Mouse BALB/c Liver, Adult) digested with Sau3A and cloned into the Bam H1 site of the λ vector EMBL3 SP6/T7 (Clontech) was used to infect the *Ecol*i host strain NM538 (Clontech). Approximately $6x10^5$ plaques were screened by hybridisation with a ^{32}P-labelled 2.5kb fragment of a rat TRH-R cDNA clone (Sellar et al., 1993) as described in Chpt. 3.2. Positive plaques were isolated following secondary screening and bacteriophage DNA was purified as previously described (Chpt. 3.3).

6.2.2 Restriction digestion of putative clones

The λ vector EMBL3 can accommodate inserts of between 8 to 22kb and to determine the size of putative TRH-R clones, each one was digested with Sac1, Xho1 or Sfi1 and electrophoresed on a 0.7% agarose gel. Molecular weight markers [λ digested with EcoR1/Hind III and pGEM (Promega), Raoul™ and λBstEII (Appligene)], with fragments ranging from 48.5kb to 36bp were run alongside the genomic digests. The approximate size of the insert was determined from the sum of the individual restriction fragments, using a standard curve (determined by measuring the distance run from the wells and the sizes of the marker fragments). Southern blots of digested genomic clones were probed with ^{32}P-end-labelled oligonucleotide probes (Chpt. 3.7.4) corresponding to different regions of

the mouse TRH-R cDNA to orientate the genomic clones with respect to the cDNA sequence.

6.2.3 PCR analysis

To identify any intronic sequences, the putative TRH-R clones were amplified by PCR (Chpt. 3.9) using sense and antisense oligonucleotides based on the published mouse cDNA sequence (Straub et al., 1990). The following PCR oligonucleotide primers were used: sense primer T1 (nucleotides -155 to -138) 5'dAACTTGGACCTATTAGCA3' and antisense primer T2 (nucleotides 473 to 490) 5'dAGCAGGAAGAACCAGAGC3'; sense primer T3 (nucleotides 1030 to 1047) 5'dCTGCTAACTACAGTGTGG3' and antisense primer T4 (nucleotides 1255 to 1273) 5'dTGCCTGAAGACATCTGTTGC3'; sense primer T5 (nucleotides 1303 to 1322) 5'dAAGATTTTCAGCAACTTACG3' and antisense primer T6 (nucleotides 1478 to 1495) 5'dCCAGTTCAAGATGTTCTT3'; sense primer T7 (nucleotides 208 to 229) 5'dGCTGATCTCATGGTCTTGGTGG3' and antisense primer T8 (nucleotides 767 to 789) 5'dGTGCTTCCTTGAAGATACTGTGC; sense primer T9 (616 to 635) 5'dCTGGCCACTGTGCTTTATGG3' and antisense primer T10 (nucleotides 858 to 878) 5'dGAGACAAATGAGTTGACAACC3'; sense primer T11 (nucleotides 873 to 892) 5'dTCTCTCCAGCCCTTTCCAGG3' and antisense primer T12 (nucleotides 1148 to 1165) 5'dAGCAGGTGTCATCAAAGG3'; sense primer T13 (nucleotides 1477 to 1494) 5'dAAGAACATCTTGAACTGG3' and the lambda vector (right arm) primer 5'dGGCCTCAAGGCCATTTAGG3'. Sizes of the amplified PCR products were compared to the rat cDNA sequence, amplified with the same primers as positive controls. Negative controls contained no DNA. Mouse, rat and human genomic DNA (Novagen) was also included, in some cases, to verify results.

6.2.4 Subcloning genomic DNA

The extremely large sizes of the genomic inserts and the λ vector arms (~20kb and ~8kb) made restriction digest and sequence analysis difficult. In some instances, smaller sections of genomic DNA such as the 3'UTR, were amplified by PCR and subcloned into the plasmid vector pCR™II (Chpt. 3.4) to facilitate analysis.

6.2.5 Genomic sequence analysis

To confirm that the isolated clones did encode the gene for the TRH-R, the clones were sequenced both manually and using the ABI automated sequencer (Chpt. 3.10). Sequencing of individual clones from the λ vector was performed manually, while sequencing of purified PCR products and subcloned genomic fragments was performed on the ABI sequencer. The computer program GeneJockey was used to analyse the sequence data.

6.2.6 The TRH-R gene in other species

Analysis of the TRH-R gene in other species was undertaken using a commercially prepared 'ZOO-BLOT' (Clontech), a Southern blot containing a panel of genomic DNAs (8μg DNA per lane) from nine eukaryotic species. The DNA had been digested with the restriction enzyme EcoR1, run on a 0.7% agarose gel and transferred to a charge-modified nylon membrane by Southern blotting. DNA was obtained from human, Rhesus monkey, Spraque-Dawley rat, mouse (BALB/c), dog, cow, rabbit, chicken and yeast (*Saccharomyces cerevisiae*). All DNA had been isolated from the kidney apart from the human, which was obtained from placenta.

The "ZOO-BLOT' was probed according to the manufacturer's instructions, with a ^{32}P random-primer labelled 1.2kb human TRH-R cDNA purified PCR fragment (5Ti/3Tp), encompassing the coding region of the TRH-R from nucleotides -18 to 1238 (see Chpt. 4.2.3).

6.3 Results

6.3.1 Isolation and restriction analysis of the mouse TRH-R gene

Several putative primary clones were initially isolated from a total of $6x10^5$ plaques screened. Restriction digestion with Sac1 revealed that clones 1, 2 and 10 were not single clones (Fig. 6.1). Following Southern blotting the digests were probed with the rat 2.5kb TRH-R cDNA (data not shown). Clones 1a, 2 and 10b failed to hybridise and were abandoned. Clones 1b and 1d were identical, but had a different restriction pattern from clone 1c (Fig. 6.1), although all three hybridised positively to the rat cDNA probe. Clones 9 (see Fig. 6.2), 4, 10a, and 12 all had different

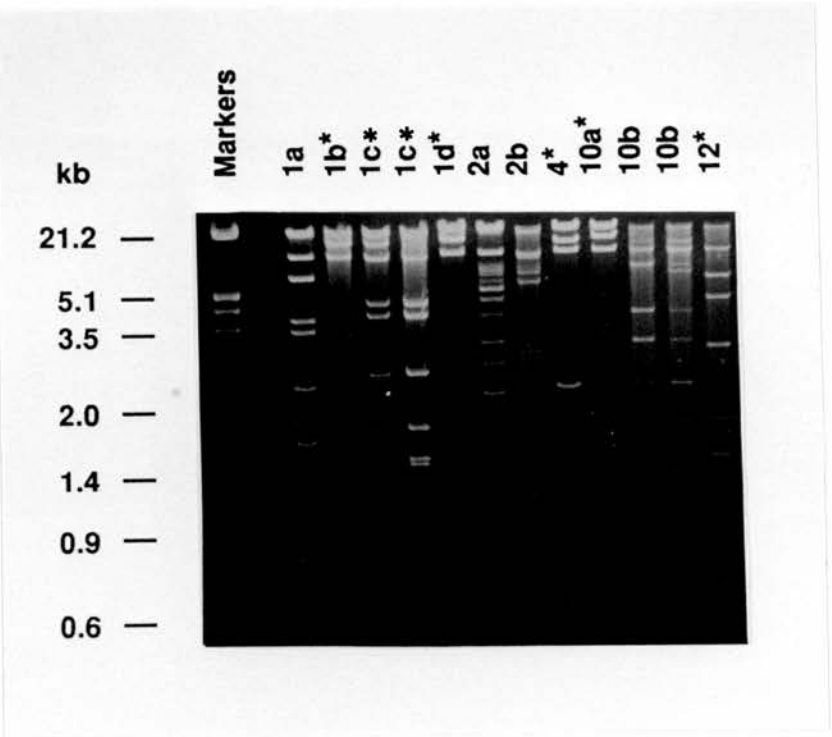


Fig. 6.1

Ethidium bromide-stained agarose gel showing Sac1 restriction digests of putative TRH-R genomic clones. (*) indicates those clones which subsequently hybridised to the rat TRH-R 2.5kb cDNA probe following Southern blotting of the agarose gel. The numbers to the left indicate standard DNA size markers (lane 1) in kilobases.

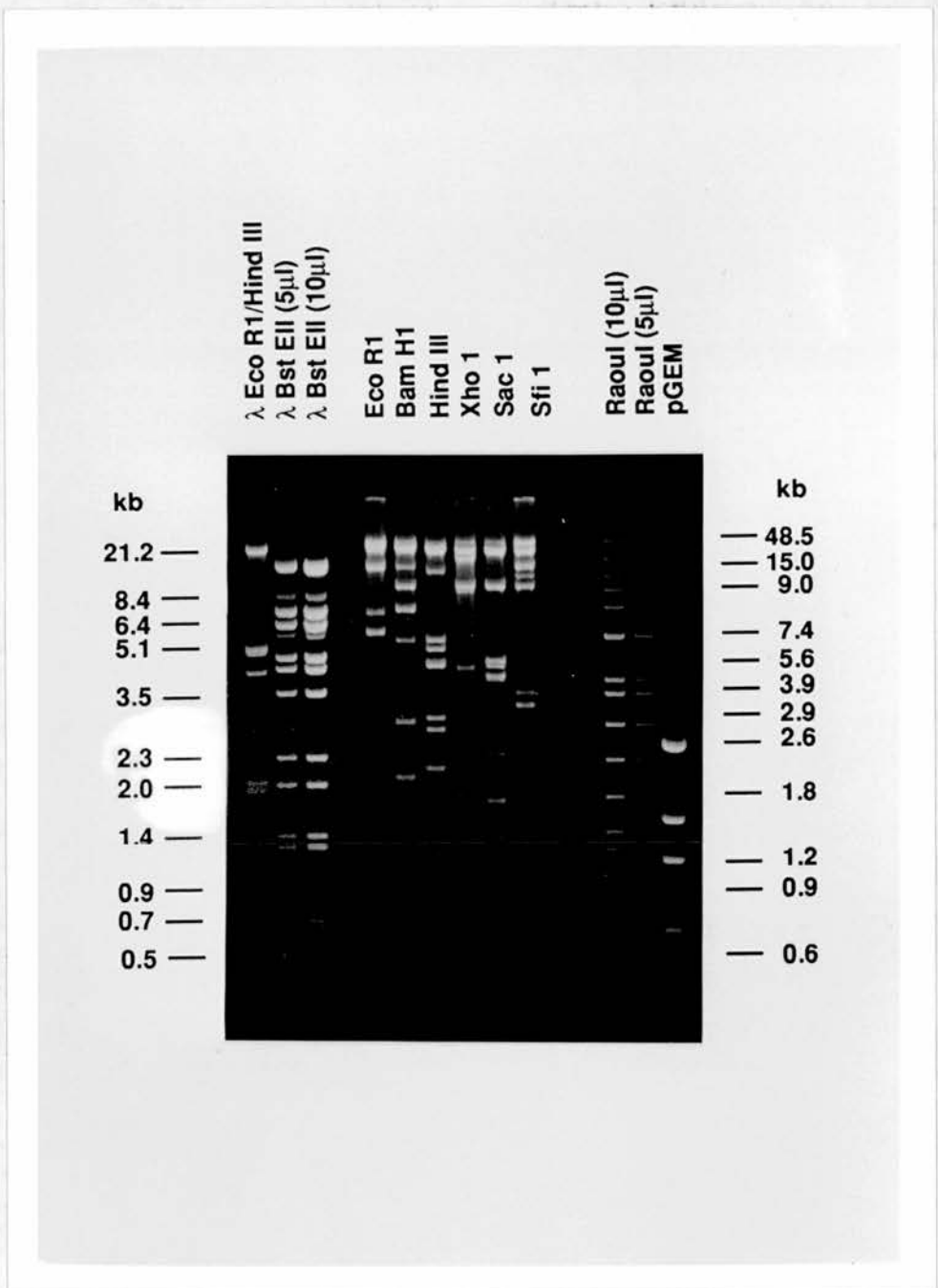


Fig. 6.2

Ethidium bromide-stained agarose gel showing restriction digestion analysis of clone 9. The first three and last three lanes contain molecular weight markers. The numbers to right and left represent the DNA size markers in kilobases.

restriction patterns and all hybridised positively to the rat TRH-R (data not shown).

Restriction digestion with Sac1 estimated the size of clone 9 as ~19kb (Fig. 6.2) with the remaining 5 clones ranging from 14kb to 17kb, [clone 1b/1d (~14kb), clone 1c (~15.5kb), clone 4 (~14kb), clone 10a (~17kb), clone 12 (~16kb)], see Fig. 6.1. Southern blotting and hybridisation with oligonucleotide probes representing different regions of the mouse cDNA (Fig. 6.3) indicated that clone 9 contained ~18.5kb of 5'UTR and the coding region as far as a Sau3A restriction site at nucleotide position 492 in the fourth transmembrane (TM) domain. The remaining 5 clones spanned the 3' portion of the coding region and the 3'UTR. Hybridisation with oligonucleotides representing the coding region from TM4 to TM6 gave confusing results and two further clones, Clone 3 and Clone 7, were eventually isolated from the mouse genomic library using a 52mer oligonucleotide, spanning nucleotides 528 to 569 of the coding region (data not shown).

6.3.2 PCR analysis of the mouse TRH-R gene

To identify intronic sequences in the mouse gene, the entire coding region of the gene was amplified by pairs of PCR primers and the sizes of the amplified genomic fragments compared to amplified rat TRH-R cDNA (Fig. 6.4). To ensure that there were no PCR or cloning artifacts, particularly between TM4 and TM6, mouse, rat and human genomic DNA was included in the PCR analysis (Fig. 6.5). An intron of approximately 645bp was detected in the 5'UTR between nucleotides -155 and +490 with primers T1 and T2. A second intron of ~1800bp was identified between nucleotides 1030 and 1273 with the PCR primers T3 and T4, and a third intron of ~350bp was disovered between nucleotides 1303 and 1495 using primers T5 and T6 (Fig. 6.4).

6.3.3 Sequence analysis of the mouse TRH-R gene

Sequence analysis of the 5'UTR region of the gene identified a consensus splice site (Ohshima & Gotoh, 1987) at position -95, separating an untranslated exon (putative Exon 1), from the coding region of the mouse gene. Just over 440bp upstream from the ATG initiation codon were manually sequenced, but no consensus promotor elements such as CAT

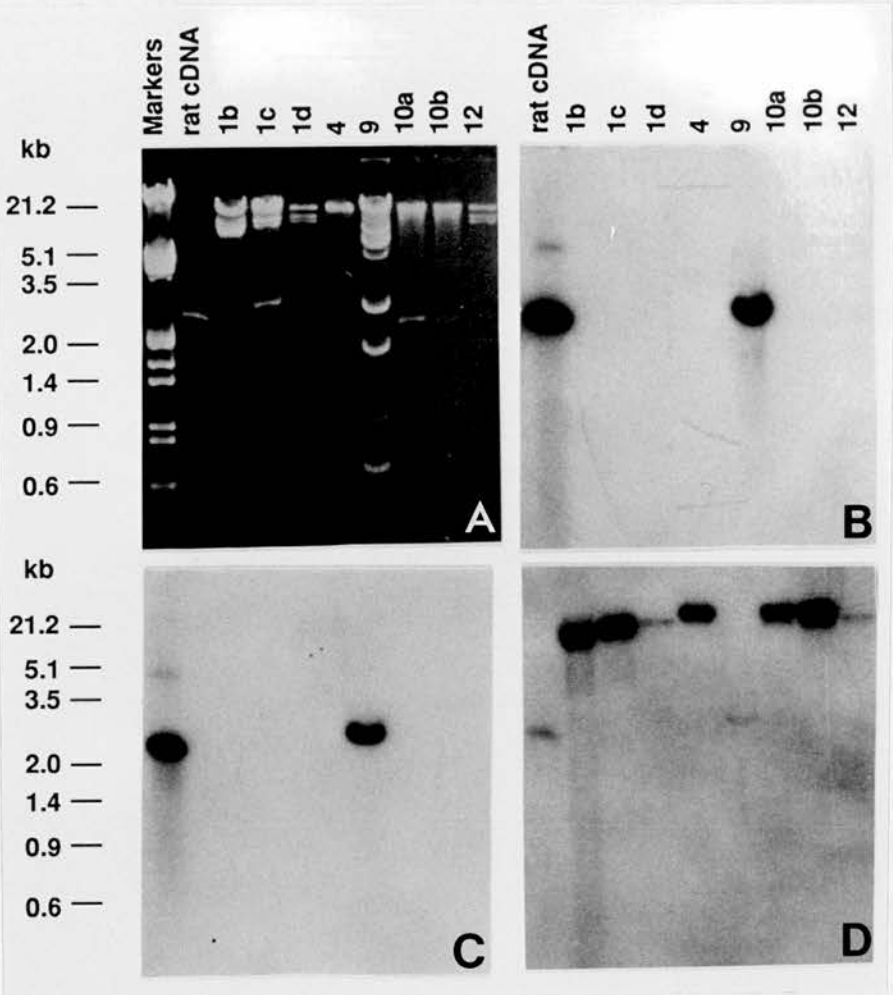


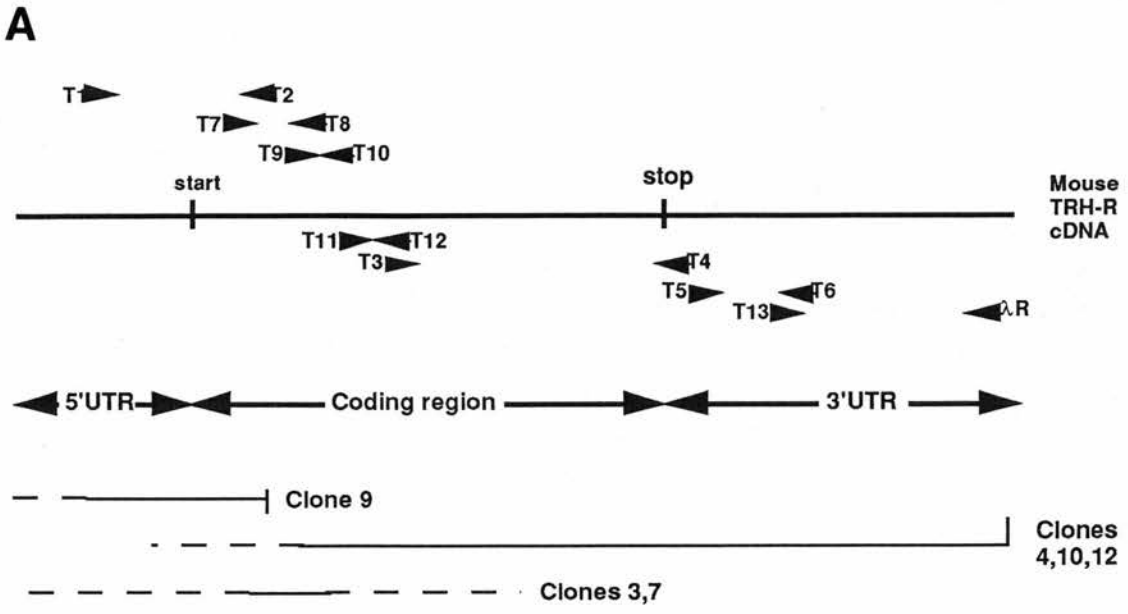
Fig. 6.3

Southern blot analysis of TRH-R genomic clones digested with BamH1 using oligonucleotide probes based on the published mouse TRH-R cDNA sequence (Straub et al., 1990). Fig. 6.3A: Ethidium bromide-stained agarose gel of BamH1-digested clones. The rat TRH-R cDNA in lane 2 acted as the positive control. Fig. 6.3B: Southern blot of gel in A, probed with an oligonucleotide representing nucleotides -155 to -138. Fig. 6.3C: Southern blot of gel in A, probed with an oligonucleotide representing nucleotides 368 to 384. Fig. 6.3D: Southern blot of gel in A, probed with an oligonucleotide representing nucleotides 1449 to 1466. The numbers to the left are standard DNA size markers in kilobases.

Fig. 6.4

(On following page)

Polymerase chain reaction (PCR) analysis of the mouse TRH-R gene indicating the presence of introns. Fig. 6.4A: Diagram indicating the positions of the PCR primers (indicated by arrows) relative to the mouse TRH-R cDNA sequence (Straub et al., 1993) used to amplify the TRH-R genomic clones. The mouse TRH-R cDNA is indicated by a thick line, PCR primers are represented by arrows, and genomic clones are represented by thin lines. The dashed lines indicate unknown sequence. Fig. 6.4B: Ethidium bromide-stained agarose gel of the PCR products amplified by the pairs of oligonucleotide primers indicated in A. Lanes 2, 5, 7, 10, 14, 17, 20 and 23 show mouse genomic clone template. Positive controls were performed using rat or human TRH-R cDNA clones as the template (lanes 3, 8, 15, 18 and 21). Negative controls (lanes 4, 6, 9, 11, 16, 19, 22 and 24) had no DNA added to the reaction. Lanes 1, 12, and 13 contain molecular size markers. The numbers in the centre are standard DNA size markers in kilobases.


A
T1
T2
T7
T8
T9
T10
start
stop
Mouse
TRH-R
cDNA
T11
T12
T3
T4
T5
T6
T13
λR
5'UTR
Coding region
3'UTR
Clone 9
Clones
4,10,12
Clones 3,7

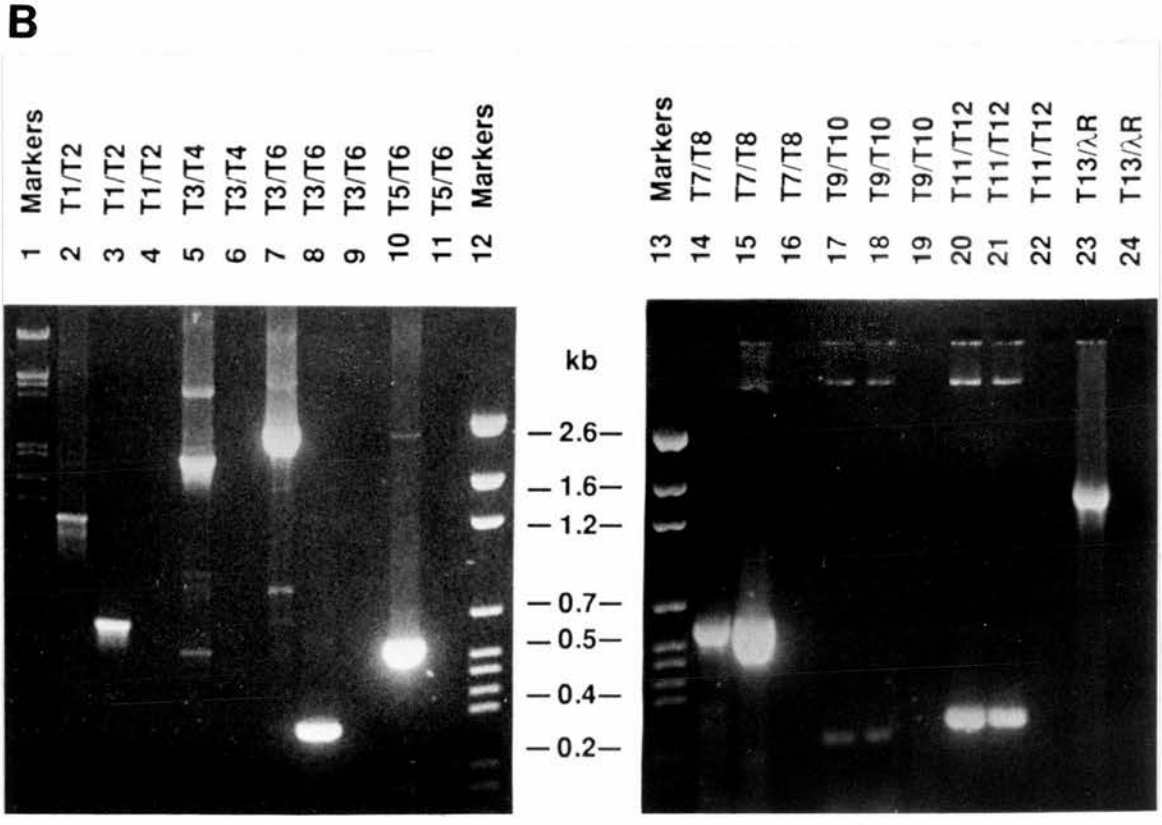
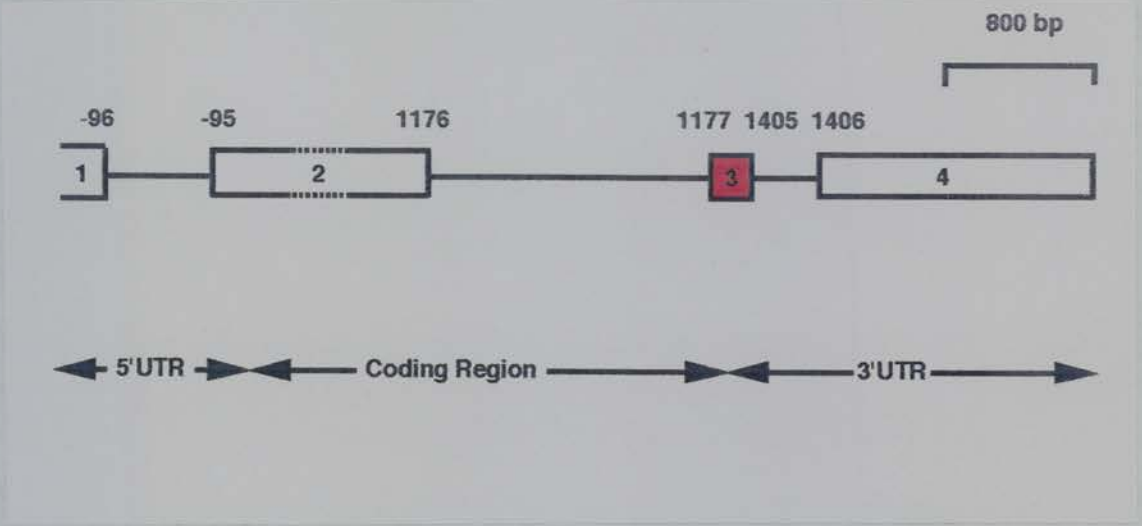

B
1 Markers
2 T1/T2
3 T1/T2
4 T1/T2
5 T3/T4
6 T3/T4
7 T3/T6
8 T3/T6
9 T3/T6
10 T5/T6
11 T5/T6
12 Markers
13 Markers
14 T7/T8
15 T7/T8
16 T7/T8
17 T9/T10
18 T9/T10
19 T9/T10
20 T11/T12
21 T11/T12
22 T11/T12
23 T13/λR
24 T13/λR
kb
2.6
1.6
1.2
0.7
0.5
0.4
0.2

Fig. 6.5

(On following page)

Polymerase chain reaction (PCR) analysis of the central region of the mouse TRH-R gene. Fig. 6.5A: Diagram indicating the structure of the mouse TRH-R gene as deduced from PCR analysis of genomic clones in Fig. 6.4. Exons are represented by boxes, the dotted line represents the region of the gene between nucleotides 208 and 878 (transmembrane domain 2 to transmembrane domain 6) amplified by the oligonucleotide primers T7 (nucleotides 208 to 229) and T10 (nucleotides 858-878). The exon corresponding to a deletion in the rat TRH-R is depicted in red. Introns are represented by lines. Fig. 6.5B: Ethidium bromide-stained agarose gel showing the PCR products amplified by the oligonucleotide primers T7 and T10. MG = mouse genomic DNA, HG = human genomic DNA, RG = rat genomic DNA. The human TRH-R cDNA (Duthie et al., 1993a) acted as a positive control. Blank = negative control with no DNA added to the PCR reaction. The numbers to the right are standard DNA size markers in kilobases.

A


800 bp
-96
-95
1176
1177 1405 1406
1
2
3
4
5'UTR
Coding Region
3'UTR

B


Markers
MG
HG
RG
1b
3
4
7
9
10
12
h cDNA
blank
Markers
kb
2.6
1.2
0.7
0.2

(CCAATCT) or TATA (TATAA) boxes were identified in the 5'UTR. However, sequencing did reveal an interesting $d(TG)_{16}.d(AG)_{21}$ dinucleotide repeat sequence 351bp upstream of the ATG codon (Fig. 6.6).

The intron/exon boundaries of the second and third introns were identified at positions 1176 and 1405 respectively (Fig. 6.5). Almost the entire coding region of the gene (392 amino acids) appeared to be contained on a single exon, Exon 2. The terminal amino acid (asparagine), stop codon (TAA) and 222bp of 3'UTR were encoded by Exon 3, separated from the rest of the coding region by the second intron (Fig. 6.6). Sequencing several times in both directions confirmed the boundaries as consensus splice junctions (Table 6.1).

The 3'UTR sequence in Exon 4 of the mouse gene revealed a region with 87% homology to the nucleotides encoding the final 20 amino acids and stop codon of the rat TRH-R_{412} (Fig.6.6 and Fig. 6.7). The 3'UTR did not contain consensus sequences for polyadenylation (AATAAA), see Fig. 6.6.

6.3.4 The TRH-R gene in other species

A commercially prepared ZOO-BLOT (Clontech) containing genomic DNA from nine eukaryotic species, digested with EcoR1, and Southern blotted, was probed with a ^{32}P-labelled PCR fragment representing the human TRH-R coding region. The probe hybridised to human, monkey, rat, mouse, dog, cow, rabbit, and chicken, but no signal could be detected in yeast (Fig. 6.8). At least two restriction fragments were identified in each species. The hybridisation signal detected in the chicken was low in comparison to other species suggesting sequence divergence in the TRH-R between mammals and birds.

6.4 Discussion

Analysis of the mouse TRH-R gene indicated at least four exons separated by three introns. The first intron (~645bp) occurred at position -95 in the 5'UTR. The second intron (~1800bp) was found at position 1176, separating the last amino acid (asparagine) and stop codon from the rest of the coding region. The third intron (~350bp) was situated between Exons 3 and 4 in the 3'UTR at position 1405 (Fig. 6.5). The mouse TRH-R

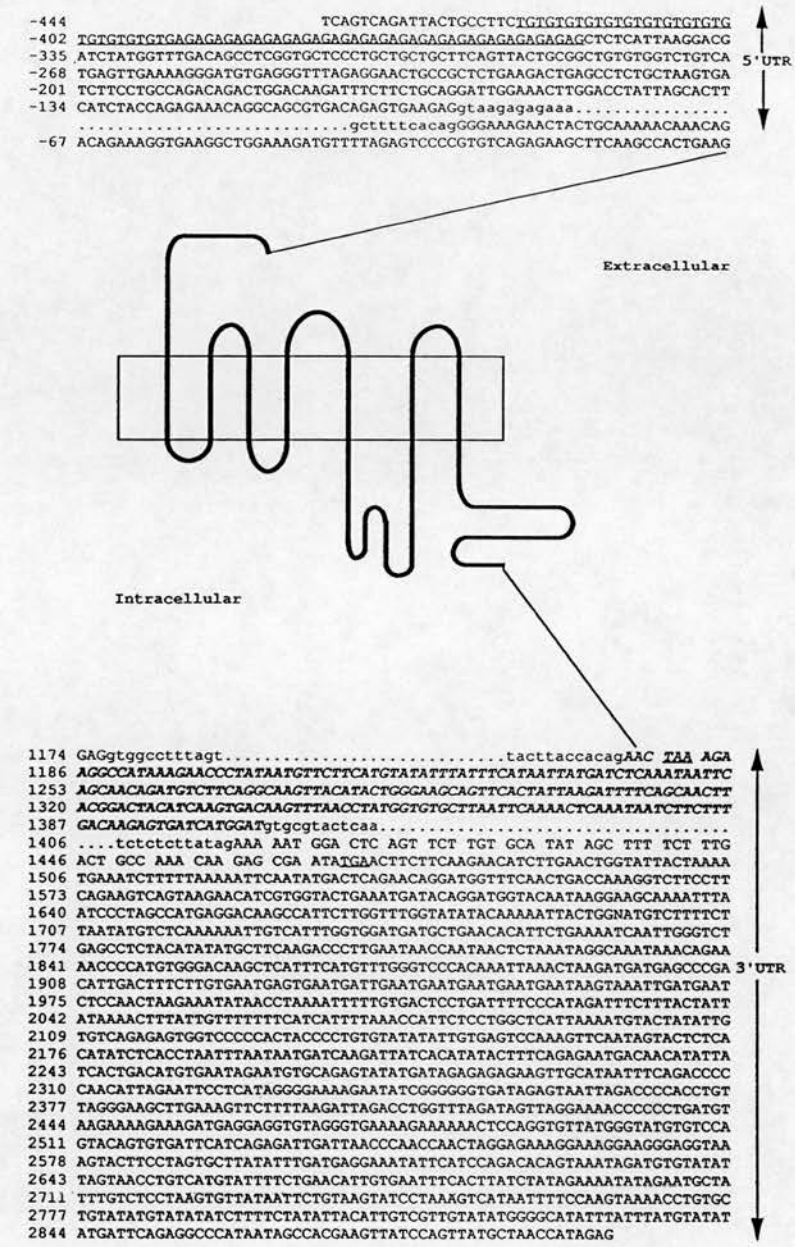


Fig. 6.6

Schematic representation of the mouse TRH-R showing the putative seven transmembrane domain topology. The nucleotide sequences for the 5' untranslated region (UTR) and 3'UTR are shown. The dinculeotide repeat in the 5'UTR is underlined. Exon 3 (corresponding to a 228bp deletion in the rat TRH-R cDNA) is shown in bold italics, and inlcudes the final asparagine codon (**AAC**) and stop codon (**TAA**) of the mouse gene. Flanking intronic sequence is represented by lower case letters and dotted lines. The mouse sequence homologous to the final 20 amino acids of the rat TRH-R COOH tail is represented as triplet codons. This sequence could be translated if Exon 3 were removed by alternative splicing. A potential stop codon (TGA) is underlined.

Donor	Acceptor	Intron Size (bp)
GAAGAG^{-96}gtaagagaga....	....ggcttttcacag^{-95} GGGAAA	~ 650
TCTGAG^{1176}gtggcctttagt....	....acttaccacag^{1177} AACTAA*	~1800
ATGGAT^{1405}gtgcgtactca....	....tctctcttatag^{1406} AAAAAT	~ 350

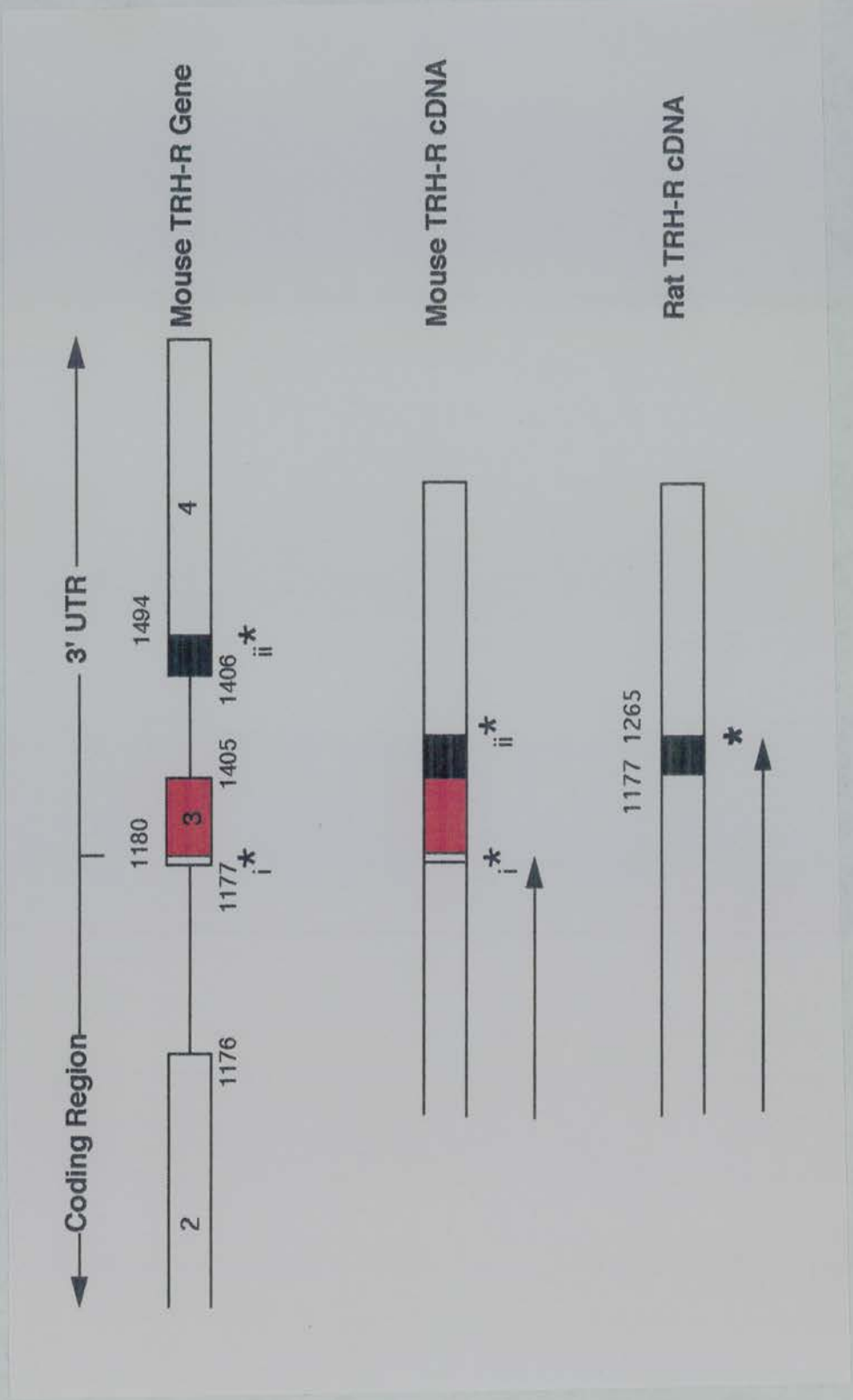
Table 6.1

Donor and acceptor splice junctions of the mouse TRH-R gene. Intronic sequences are represented by lower case letters and dotted lines. The stop codon TAA is indicated by (*).

Fig. 6.7

(On following page)

Diagrammatic representation of the COOH terminus of the TRH-R gene. Introns are represented by lines, exons are represented as boxes, stop codons are represented by (*), arrows indicate the coding regions in mouse and rat TRH-R cDNA sequences. The red box indicates Exon 3 of the mouse TRH-R gene which corresponds exactly to a 228bp deletion in the rat TRH-R cDNA. The black box in Exon 4, from nucleotides 1406 to 1494 in the mouse gene has 87% homology with the rat cDNA sequence from nucleotides 1177 to 1265, also shown as a black box. Alternative splicing of the mouse Exon 3 (red box) would remove the first stop codon (i*) and extend the 393 amino acid mouse receptor protein into Exon 4. This would result in a 412 amino acid receptor with 95% homology to the rat TRH-R.


Mouse TRH-R Gene
Mouse TRH-R cDNA
Rat TRH-R cDNA
Coding Region
3' UTR
2
3
4
1176
1177
1180
1405
1406
1494
i
ii
1177
1265

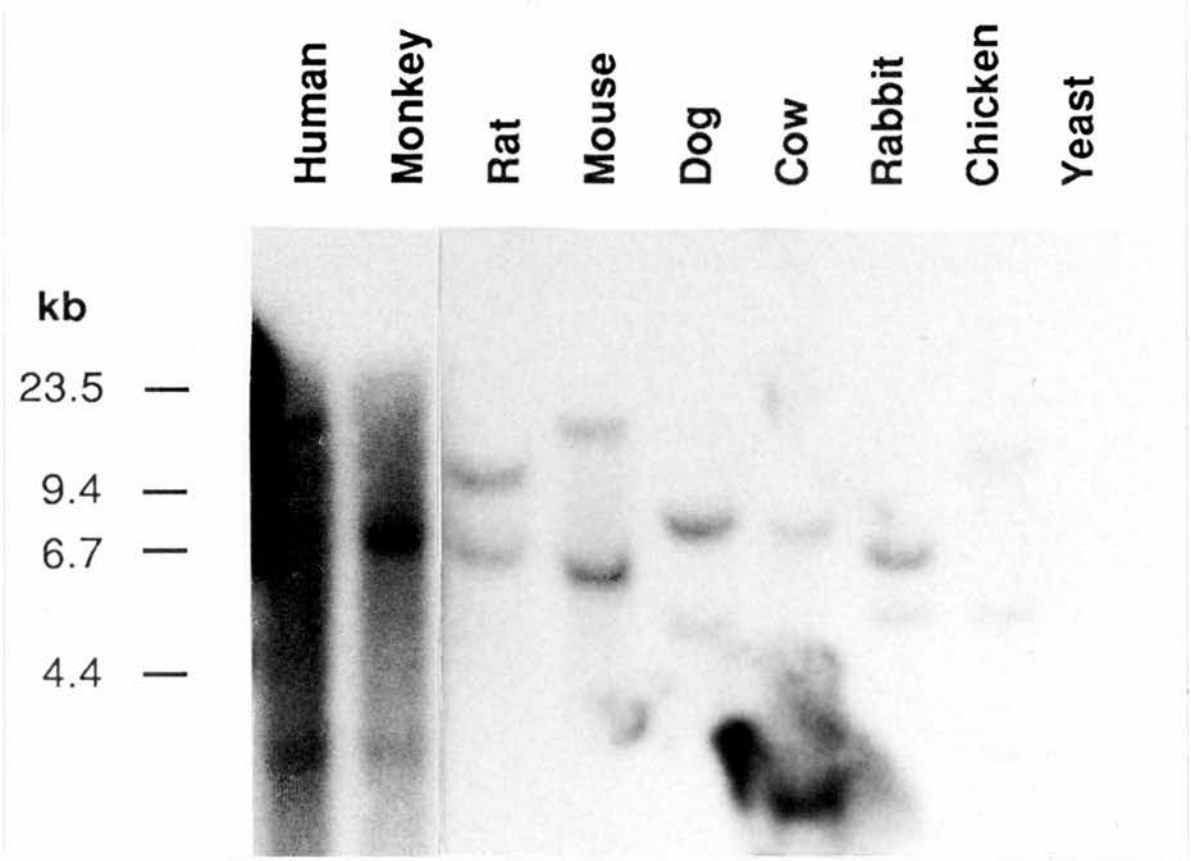


Fig. 6.8

Southern blot hybridisation analysis of a panel of genomic DNAs isolated from several species (Clontech - ZOOBLOT). The commercially prepared blot contained genomic DNA digested with EcoR1. The blot was probed with a ^{32}P-labelled human TRH-R PCR fragment representing the coding region of the human TRH-R from nucleotides -18 to 1238. The numbers to the left are standard DNA size markers in kilobases.

gene therefore belongs to an increasing number of GPRs that contain introns, for examples see Chpt 2.6.32.

The 5'UTR of the mouse TRH-R gene

Exon 1 was untranslated but did not appear to contain consensus sites for promotor elements such as CAT or TATA boxes, suggesting that the transcriptional start site was situated further upstream than the 440bp sequenced. There may be further exons upstream of putative Exon 1 as was found for the angiotensin II receptor (Curnow et al., 1992). Exon 1 did contain however, a repeat sequence $d(TG)_{16}.d(AG)_{21}$ situated 351bp upstream from the ATG initiation codon (Fig. 6.6). Alternating purine-pyrimidine (APP) sequences such as the TG repeat, may allow DNA to assume its lowest energy confirmation as Z-DNA (Rich et al., 1984). DNA is polymorphic in structure, and can exist in conformations other than the characteristic, right-handed helical B-DNA. Left-handed Z-DNA is the best characterised of these alternative structures and is often found in controlling regions of genes such as promotors and enhancers, (Schroth et al.,1992). Alteration between B-DNA and Z-DNA requires negative supercoiling of the helix which has been implicated in the modulation of gene replication, transcription and recombination (Liu & Wang, 1987).

Genbank/EMBL database searches found at least twenty genes with a $d(TG)_n.d(AG)_n$ repeat, either in the same, or the reverse $d(CT)_n.d(CA)_n$ orientation, and many of the repeats were localised to control regions. Regions containing similar dinucleotide repeats in both the rat prolactin gene 5'UTR (Naylor & Clark, 1990) and the rat ceruloplasmin gene 5'UTR (Fleming et al., 1992) were found to exert negative effects on gene transcription, whilst a $d(TG)_n.d(AG)_n$ repeat was located in a 3' tissue specific enhancer region of the CD3-δ gene (Georgopoulos et al., 1988). The rat substance P receptor, a member of the GPR superfamily, also encoded a $d(GT)_n$ repeat in the 5'UTR and a $d(CT)_n.d(CA)_n$ repeat in the 3'UTR (Yokota et al., 1989), although its function is unknown. Functional studies with reporter genes should help to determine whether the $d(TG)_{16}.d(AG)_{21}$ repeat in the mouse TRH-R 5'UTR plays a regulatory role in controlling gene transcription.

Exon 3 of the mouse TRH-R corresponds to a deletion in the rat TRH-R cDNA

The mouse TRH-R cDNA (Straub et al., 1990) encodes a putative protein of 393 amino acids, and the long form of the rat TRH-R encodes a receptor protein of 412 residues (Zhao et al., 1992; de la Peña et al., 1992a). Although the rat cDNA encodes a longer protein, it actually contains a 228bp deletion relative to the mouse sequence. This 228bp deletion in the rat corresponds exactly to Exon 3 of the mouse TRH-R gene, which contains the stop codon (TAA) for the mouse receptor protein. Deletion of this region in the rat sequence removes the stop codon resulting in a longer protein of 412 amino acids encoding 20 amino acids after residue 392, as compared to the the mouse TRH-R cDNA, which encodes only one residue after this point (Fig. 6.7).

Interestingly, the nucleotide sequence for the final 20 residues and stop codon of the rat receptor is found encoded in the mouse gene at the beginning of Exon 4 (Fig. 6.7). Splicing out Exon 3 of the mouse TRH-R gene would remove the terminal amino acid (asparagine) and the stop codon allowing the mouse gene to read directly into Exon 4. The nucleotide sequence for the final 20 residues would then be in-frame with the rest of the coding region, resulting in a mouse TRH-R protein of 412 amino acids with 95% homology to the rat TRH-R_{412} sequence (Fig. 6.7). To date, only one form of the TRH-R has been isolated from the mouse, and although there is no direct evidence, it is possible that the mouse gene could produce at least two forms of TRH-R mRNA encoding different COOH tails, as a result of alternative splicing. Alternative splicing has been demonstrated in other GPR genes including the dopamine D2 receptor (Giros et al., 1989; dal Toso et al., 1989; O'Malley et al., 1990, Mack et al., 1991), the angiotensin II receptor (Curnow et al., 1992), the PACAP receptor (Spengler et al., 1993) and the prostaglandin EP3 receptor (Namba et al., 1993).

Until recently, functional signficance for such splicing events had not been demonstrated, however alternative splicing in the third cytoplasmic loop (CL3) of both the D2 receptor (Montmayeur et al., 1993), (see Chpt. 7) and the PACAP receptor (Spengler et al., 1993) were shown to affect G-protein coupling and second messenger activation. Of relevance to the TRH-R gene, probable alternative splicing of the

prostaglandin EP3 receptor occurs at the 3' end of the COOH tail (Namba et al., 1993). There are at least four isoforms of the EP3 receptor, all identical up to amino acid 358 and differing only at the 3' end of their COOH tails in a similar manner to the TRH-R subtypes. These isoforms were shown to have different specificities for coupling to G-proteins, but identical ligand binding characteristics and it is possible that further studies with the different TRH-Rs may produce similar findings.

Evolution of the TRH-R gene

Results obtained from Southern hybridisation of the human TRH-R cDNA with a panel of genomic DNAs froma variety of species (ZOOBLOT) showed a certain conservation of the TRH-R gene between mammals, and to a lesser extent, birds, as the hybridisation signal was much lower in the chicken. No signal was detected in yeast, although PCR analysis of the TRH-R gene in different species (Murray-McIntosh et al., 1993) did obtain a positive result from yeast.

Mouse genomic DNA revealed two restriction fragments hybridising to the human TRH-R probe indicating the presence of an EcoR1 site within the TRH-R gene (Fig. 6.8). No EcoR1 sites have been reported within the coding region of the mouse TRH-R (Straub et al., 1990), nor were any EcoR1 sites found within the 3 introns of the mouse TRH-R gene. One possibility is that an unidentified intron exists within the gene. This is unlikely as repeated PCR experiments with different sets of primers failed to discover any introns other than at the COOH tail, and no introns have been found within the coding region by others (M.C. Gershengorn, personal communication, see Appendix IV). Alternatively, the two bands could represent a restriction fragment polymorphism if the mouse cDNA (Straub et al., 1990) was cloned from a different species than the mouse gene, which was cloned from a BALB/c mouse.

The rat gene has been analysed by PCR (de la Peña et al., 1992b) and no introns other than the retained intron in the coding sequence, have been described. There do not appear to be any EcoR1 sites within the coding region of the rat TRH-R, but again, two restriction fragments were obtained from the ZOOBLOT, raising similar possibilities as suggested for the mouse gene above.

The human TRH-R gene has recently been isolated by Yamada et al. (1993) and has one intron in the third cytoplasmic loop. No intron was described at the breakpoint residue 392 for the human TRH-R gene (Yamada et al., 1993) or the rat gene (de la Peña et al., 1992b). Three EcoR1 restriction fragments were detected in human (Fig. 6.8) and though there are no EcoR1 sites within the coding region of the human TRH-R, these restriction sites may occur within the intron discovered in the third cytoplasmic loop (Yamada et al., 1993). The TRH-R genes reported for the three species, mouse, rat and human are quite different in organisation (Fig. 6.9) and appear have diverged to a greater extent during evolution than have the dopamine D2-R genes described in Chpt. 7.

TRH-R subtypes in vitro and in vivo

It has been shown that TRH-R-stimulation of TSH in thyrotrophs and prolactin in lactotrophs occurs by different Ca^{2+}-mediated mechanisms (Geras et al., 1982) which suggests that subtypes of the mouse TRH-R may exist and have yet to be discovered. An attempt to isolate other forms of the mouse TRH-R from a lactotroph, rather than a thyrotroph, cell line may provide interesting results.

The study of receptors cloned from tumour-derived cell lines and the analysis of their second messenger systems *in vitro* may not reflect the situation *in vivo*. Results obtained from transfection studies in heterologous cell lines, such as COS-1 cells or 293 kidney fibroblast cells, do not always agree with results obtained from cell lines expressing endogenous receptors, such as GH3 cells, or from results obtained *in vivo*. Yamada et al. (1992) found that TRH-R mRNA was downregulated by thyroid hormones *in vivo*, but found no effect on downregulation of the mRNA in GH3 cells, although TRH-Rs themselves were downregulated in both systems. Ultimately, it will be necessary to study the regulation of the TRH-R gene in an *in vivo* system. It may be possible to target the mouse TRH-R gene to thyrotrophs or lactotrophs using tissue-specific promotor elements such as the TSH or prolactin promotors, and then to study the regulation of the TRH-R gene in transgenic mice.

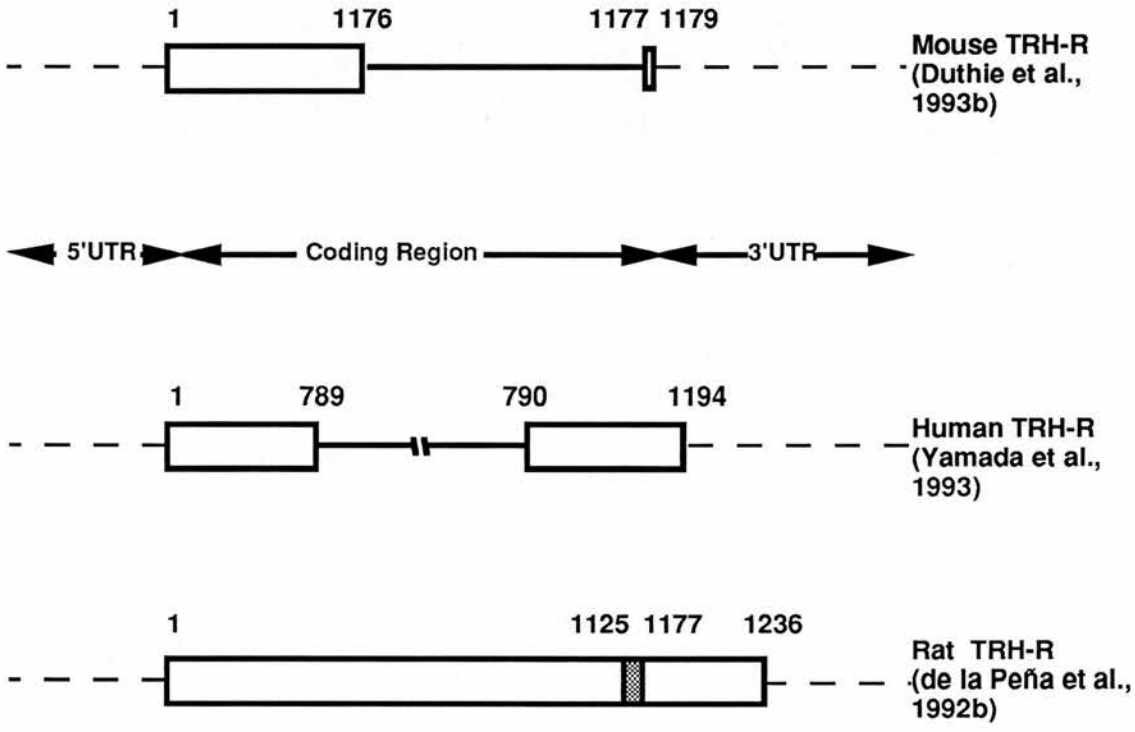


Fig. 6.9

Diagram comparing the reported organisation of the coding regions of the mouse, human and rat TRH-R genes. Boxes indicate coding sequence, solid lines indicate intronic sequence, the shaded box represents the 'retained intron' in the rat sequence, dashed lines indicate untranslated regions.

6.5 Summary

In conclusion, this chapter has reported the cloning and characterisation of a gene encoding the mouse TRH-R focusing on the COOH tail region of the receptor. Three introns of ~645bp, ~1800bp and ~350bp were discovered within the gene. The putative first exon was untranslated and almost the entire coding region of the receptor appeared to be contained on Exon 2 except for the final amino acid and stop codon found on Exon 3. The remainder of Exon 3 and Exon 4 encoded 3' untranslated sequence. The lack of promotor elements or a poly A tail allows the possibility of further exons up or downstream of those reported. The mouse TRH-R cDNA is highly homologous to the rat and human TRH-Rs except at the COOH tail where the terminal amino acids show sequence divergence after residue 392. The rat TRH-R_{412} subtype encodes 20 amino acids after this point and the nucleotide sequence for these residues was found to be encoded by Exon 4 of the mouse gene. Exon 3 of the mouse TRH-R corresponds exactly to a 228bp deletion in the rat cDNA. A splicing event in this region of the mouse gene would produce the same deletion in the mouse TRH-R as occurs in the rat TRH-R_{412}, resulting in a mouse TRH-R_{412} with 96% homology to the rat TRH-R_{412} receptor. It is possible that more than one form of mouse TRH-R may be produced from the TRH-R gene by alternative splicing of the mRNA.

7 Structural analysis of the mouse dopamine D2 receptor gene

7.1 Introduction

Dopamine was initially considered to be only a precursor in the catecholamine pathway, however it is now known to be an important neurotransmitter and neurohormone in its own right, exerting its effects through multiple dopamine receptor subtypes (Kebabian & Calne, 1979; Civelli et al., 1991; Civelli et al., 1993). In the anterior pituitary gland, dopamine plays an important role in reproduction by inhibiting the release of prolactin from lactotrophs (Chpt 2.5), and dopamine agonist therapy can be used to treat prolactin-secreting adenomas of the pituitary (Bevan et al., 1992). Dopamine is also associated with neurological disorders such as Parkinson's disease, schizophrenia, susceptibility to drug abuse and alcoholism. The involvement of dopamine in the pathophysiology of these neuronal and endocrine disorders has resulted in intensive research into the biochemistry, pharmacology and, more recently, the molecular biology of the dopamine receptors.

By 1979, the dopamine receptors had been classified as D1-like and D2-like receptors (Kebabian & Calne, 1979) on the basis of their biochemistry and pharmacology (Chpt 2.4.8) however, the advent of molecular cloning techniques revealed multiple dopamine receptor subtypes. Dopamine receptors belonged to the G-protein-coupled receptor (GPR) family, as both D1-like and D2-like receptors were known to interact with G-proteins to induce second messenger systems (Vallar & Meldolesi, 1989; Civelli et al., 1993). The dopamine D2 receptor (D2-R) was the first to be cloned in 1988 (Bunzow et al., 1988) using an approach based on the transmembrane sequence homology observed between members of the GPR family. The hamster β-adrenergic receptor (βAR) was used as a hybridisation probe to isolate a 415 amino acid D2-R from a rat brain cDNA library. The molecular cloning of the rat D2-R cDNA launched a new approach to dopamine receptor research by making it possible to isolate receptor subtypes that could not be detected by ligand-binding techniques. The subsequent characterisation of four more

dopamine receptor genes has shown that the dopamine receptor family is indeed more diverse than the original D1/D2 receptor classification.

Soon after the isolation of the rat brain D2-R cDNA, a second isoform of the D2-R was detected in rat pituitary (Eidne et al., 1989), rat brain (Rao et al., 1990), bovine brain (Chio et al., 1990), human brain (Grandy et al., 1989) and human pituitary (Dal Toso et al., 1989). This second form of the D2-R (D2-R_{444}) was longer by 29 amino acids which were encoded on a separate exon. This extra exon was inserted in the third cytoplasmic loop of the receptor, a domain that had been implicated in receptor coupling to G-proteins (Dixon et al., 1988). The two forms of the D2-R were shown to be alternatively spliced versions of the same gene (Giros et al., 1989; Monsma et al., 1989; Dal Toso et al., 1989; O'Malley et al., 1990) and it was proposed that this event was possibly a selective mechanism for coupling to different G-proteins and activating different signalling pathways. There was considerable evidence that the D2-R could couple to the phosphoinositide (PI) pathway as well as to the cAMP pathway (Kebabian & Calne,, 1979; Senogles et al., 1987; Ohara et al., 1988; Vallar & Meldolesi, 1989; de Keyser et al., 1989; Elazar et al., 1989), lending support to this theory.

Expression studies with the long (D2-R_{444}) and short (D2-R_{415}) forms of the dopamine D2-R demonstrated that the two isoforms appeared to coexist in all regions of the brain and pituitary studied. The relative proportions of the two isoforms varied in different reports, but all agreed that the D2-R_{444} was the most abundant transcript in the pituitary (Giros et al., 1989; Dal Toso et al., 1989; O'Malley et al., 1989). The genomic structure of the rat (Giros et al., 1989; O'Malley et al., 1990) was then characterised and was found to be encoded by 8 exons. The first exon was untranslated and was separated from the coding region of the gene by a large (>25kb) intron. Chromosome walking techniques were unable to close the gap between the first and second exons, and the size of this first intron was undetermined at this time. The remaining 7 exons were clustered together, spanning approximately 13kb of the genome and the alternatively spliced 29 amino acids were encoded by exon 6.

Linkage of the dopamine D2-R to several neuronal and endocrine human pathologies underlines the importance of obtaining detailed information regarding the structure-function relationships of this gene,

and the regulation of its two isoforms. Ultimately, the creation of transgenic animal models of human D2-R-related diseases may be possible. As discussed in Chpt. 6 for the mouse TRH-R gene, the eventual aim of this project was to examine the functioning of the D2-R gene using transgenic mice. Constructs created from the D2-R gene driven by the prolactin promotor could be used in an attempt to specifically target lactotrophs of the anterior pituitary. As the first step in this process, the cloning and characterisation of the mouse D2-R gene was undertaken, the mouse being the species of choice for the creation of transgenic animal models.

7.2 Materials and methods

The mouse D2 receptor (D2-R) gene was cloned from a commercially available genomic library as described in the general materials and methods section in Chpt. 3. This section will, therefore, only give specific details regarding the library, host cells and probe used to isolate the gene.

7.2.1 Screening an EMBL3 mouse genomic library

A commercially prepared library of mouse genomic DNA (Mouse BALB/c Liver, Adult) cloned into the Bam H1 site of the λ vector EMBL3 SP6/T7 (Clontech) was used to infect the *Ecoli* host strain NM538 (Clontech). Approximately $6x10^5$ plaques were screened with a ^{32}P-labelled 3kb EcoR1-digested fragment of the rat dopamine D2-R cDNA, isolated as described previously (Eidne et al., 1989). The probe was double-labelled with both $\alpha^{32}P$ dCTP and $\alpha^{32}P$ dATP. Positive plaques were isolated and purified as described in Chpt. 3.3.

7.2.2 Restriction digestion of positive clones

To determine the size of each clone, purified bacteriophage DNA was digested with the restriction enzymes Xho1 and Sac1, which released the genomic insert. The digested clones were electrophoresed on a 0.7% agarose gel (Chpt. 3.7), Southern blotted (Chpt. 3.7.4) and reprobed with the rat D2-R cDNA probe to confirm the presence of positively hybridising clones. The λ vector EMBL3 can accommodate inserts ranging from 8 to 22kb and high molecular weight markers (λ EcoR1/Hind111 [Promega])

ranging from 21.2kb to 125bp, were run alongside the digested clones. A standard curve was used to determine the sizes of the restriction fragments. In an attempt to map the gene, one of the positive clones was chosen for further digestion with BamH1, EcoR1 and Xho1, Southern blotted and probed with ^{32}P-end-labelled oligonucleotides (Chpt. 3.8.5) corresponding to each of the 8 D2-R exons in the rat. The oligonucleotides were chosen by referring to the published rat D2-R genomic structure (O'Malley et al., 1990).

7.2.3 PCR analysis

To compare the mouse D2-R gene with the rat D2-R gene, the genomic clones were amplified by PCR (Chpt. 3.9) using primers that would amplify the introns described by O'Malley et al. (1990). The following primers were used: Intron 2: sense primer 2B (nucleotides 157 to 173) 5'dGTGCTGGTGTGCATGGC3' and antisense primer 3A (nucleotides 311 to 328) 5'dAGATGTCACAGTGAATCC3'; Intron 3: sense primer 3B (nucleotides 323 to 340) 5'dACATCTTTGTCACTCTGG3' and antisense primer 4A (nucleotides 480 to 497) 5'dATGGTGAAGGACAGGACC3'; Intron 4: sense primer 4B (nucleotides 430 to 447) 5'dACAGCTACAGCTCCAAGC3' and antisense primer 5A (nucleotides 615 to 632) 5'dTTGATATAGACCAGCAGG3'; Intron 5: sense primer 5B (nucleotides 565 to 582) 5'dTTCGTGGTCTACTCCTCC3' and antisense primer 6A (nucleotides 744 to 765) 5'dAACGGTGCAGAGTTTCATGTCC3'; Intron 6: sense primer 6B (nucleotides 742 to 762) 5'dGAGGACATGAAACTCTGCACC3' and antisense primer 7A (nucleotides 1085 to 1102) 5'dTCTGCTGGGAGAGCTTCC3'; Intron 7: sense primer 7B (nucleotides 1025 to 1042) 5'dTTGAGATCCAGACCATGC3' and antisense primer 8A (nucleotides 1157 to 1174) 5'dTGAAGAAGGGCAGCCAGC3'.

7.2.4 Genomic sequence analysis

The purified PCR fragments (Chpt. 3.9) were sequenced several times in both directions using the automated ABI DNA sequencer (Chpt. 3.10) to confirm the positions of the splice junctions at the intron/exon boundaries. The computer program GeneJockey was used to analyse the sequence data.

7.3 Results

7.3.1 Isolation and restriction analysis of the mouse D2-R gene

Fourteen positively hybridising clones were initially isolated from the mouse λ EMBL3 genomic library, but only five hybridised to the D2-R cDNA probe following restriction digestion and Southern blotting (Fig. 7.1). Restriction digestion analysis of purified DNA with Xho1 and Sac1 indicated that all five clones were approximately 12kb in size (Fig. 7.2). Southern blots of the digested clones were probed with oligonucleotides representing each of the eight exons as reported in the rat D2-R gene (O'Malley et al., 1990) and results showed that none of the clones represented Exon 1 of the D2-R. Clone 7 consisted of Exon 2 and approximately 10kb of intron 1. The other four clones hybridised positively to oligonucleotides for Exons 2 to 8 (Fig. 7.3). A restriction map of the mouse D2-R gene as encoded by these five clones was derived from digestion with BamH1, EcoR1, and Xho1 (Fig. 7.3).

7.3.2 PCR analysis of the mouse D2-R gene

PCR amplification of the positive clones indicated that the mouse D2-R gene is organised in the same way as the rat and human genes (Fig. 7.4), intron sizes varying only slightly (Table 7.1). Intron 2 proved too large (>4kb) to amplify by PCR and its size was determined from restriction digestion analysis. Exons 2 to 8 of the mouse D2-R gene were clustered in approximately 11 to 12kb of the genome.

7.3.3 Sequence analysis of the mouse D2-R gene

The intron/exon boundaries of the purified PCR fragments were sequenced and were found in the same positions as the rat (O'Malley et al., 1990) and human (Gandelman et al., 1991) D2-R genes. Like the rat, but unlike the human gene, the mouse D2-R had a variant donor splice site between Exons 4 and 5, with a gc instead of the more usual gt dinucleotide (Ohshima & Gotoh, 1987) as the first two nucleotides of the intron (Table 7.1). The initiation codon was found in Exon 2, following 30bp of 5' untranslated region (5'UTR) and the termination codon occurred at

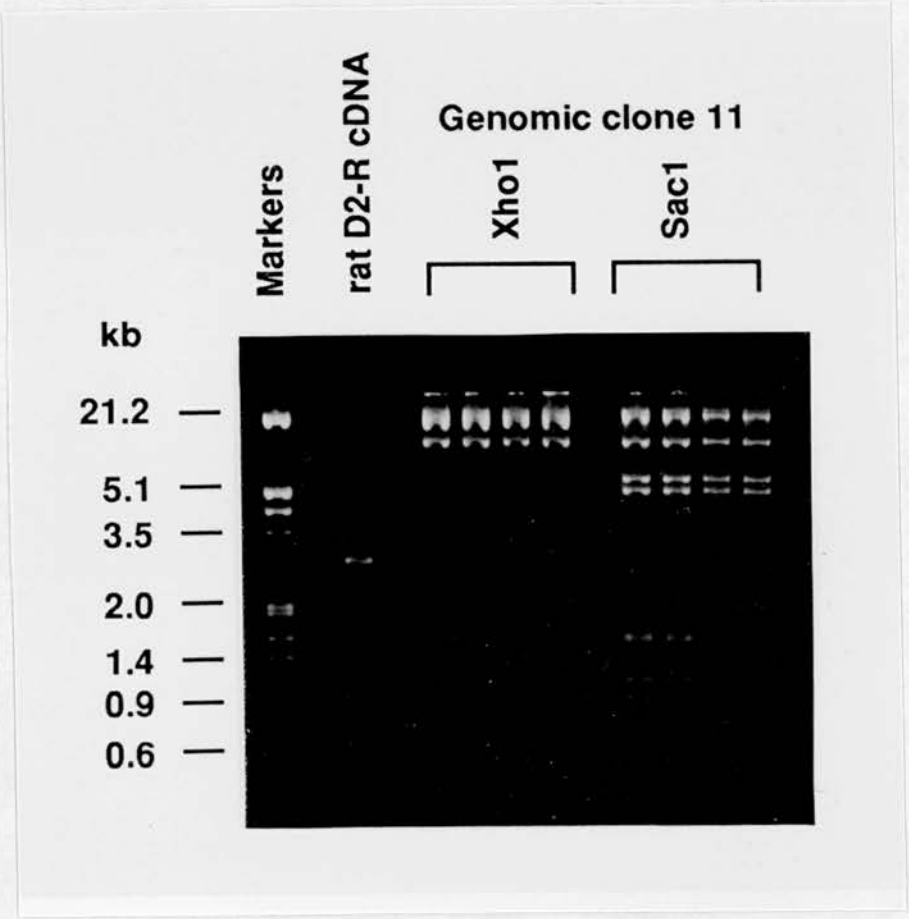


Fig. 7.1A

Restriction digestion and Southern blot analysis of one dopamine D2-R genomic clone. Fig. 7.1A: Ethidium bromide-stained agarose gel showing clone 11 digested with Xho1 and Sac1 to release the genomic insert from the λ EMBL3 vector. The numbers to the left indicate standard DNA size markers in kilobases. Fig. 7.1B showing the Southern blot is on the following page.

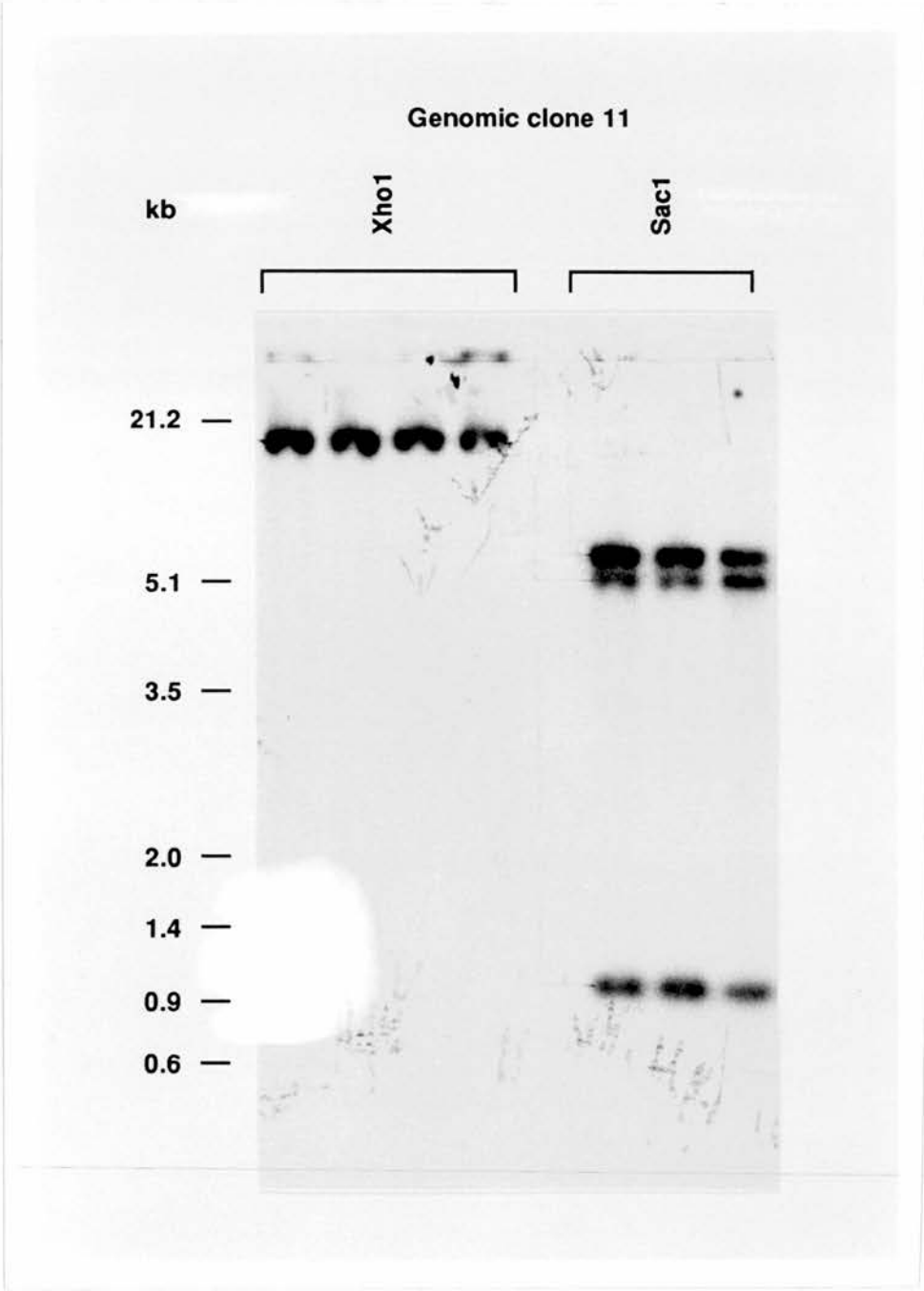


Fig. 7.1B

Restriction digestion and Southern blot analysis of one dopamine D2-R genomic clone. Fig. 7.1B: Autoradiograph of Southern blot hybridisation of clone 11. The DNA was digested as indicated in Fig. 7.1A, blotted and probed with the ^{32}P-labelled 3kb rat dopamine D2-R cDNA (Eidne et al., 1989). The numbers to the left indicate standard DNA size markers in kilobases.

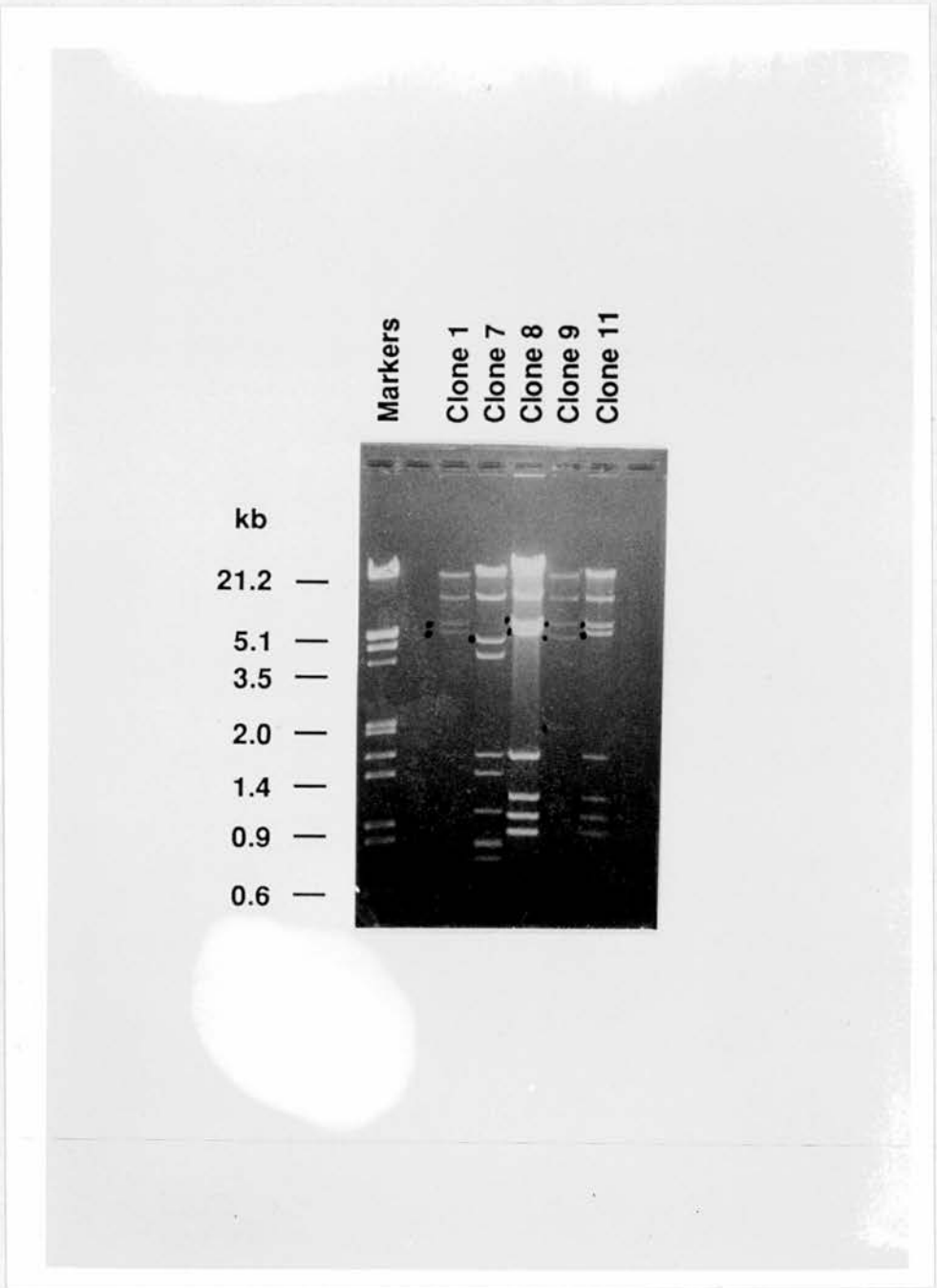


Fig. 7.2

Ethidium bromide-stained agarose gel showing the five dopamine D2-R genomic clones digested with Sac1. The numbers to the left indicate standard DNA size markers in kilobases.

A

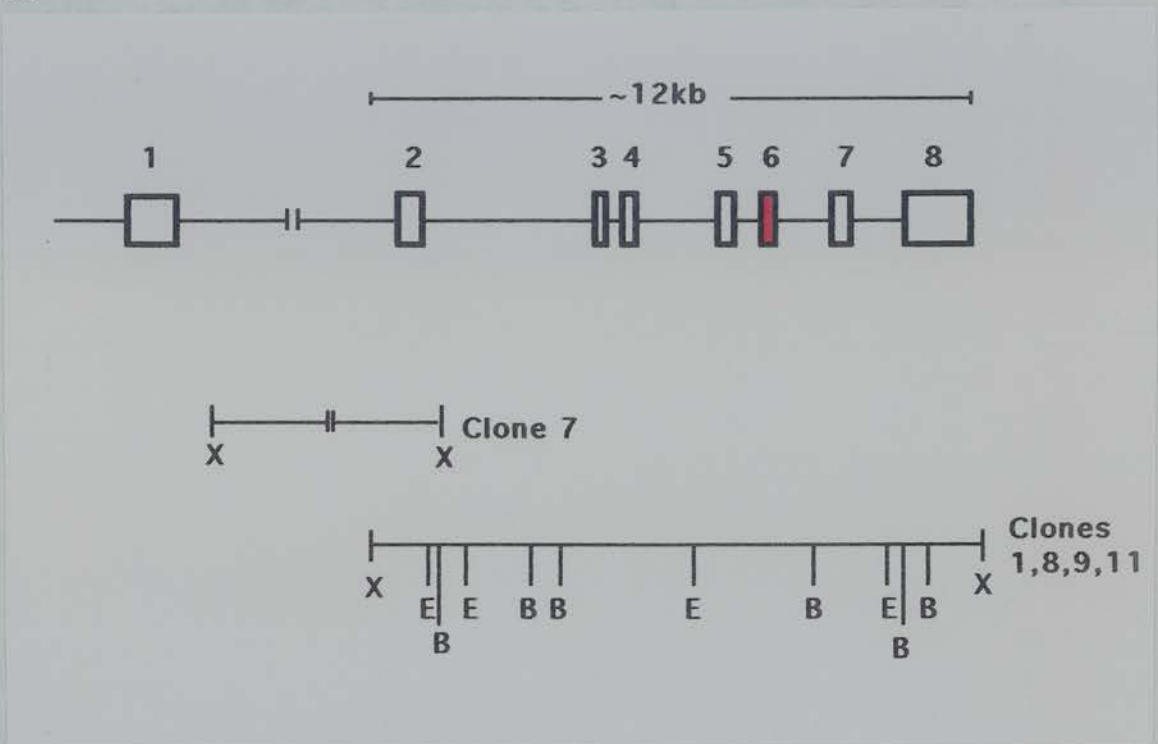
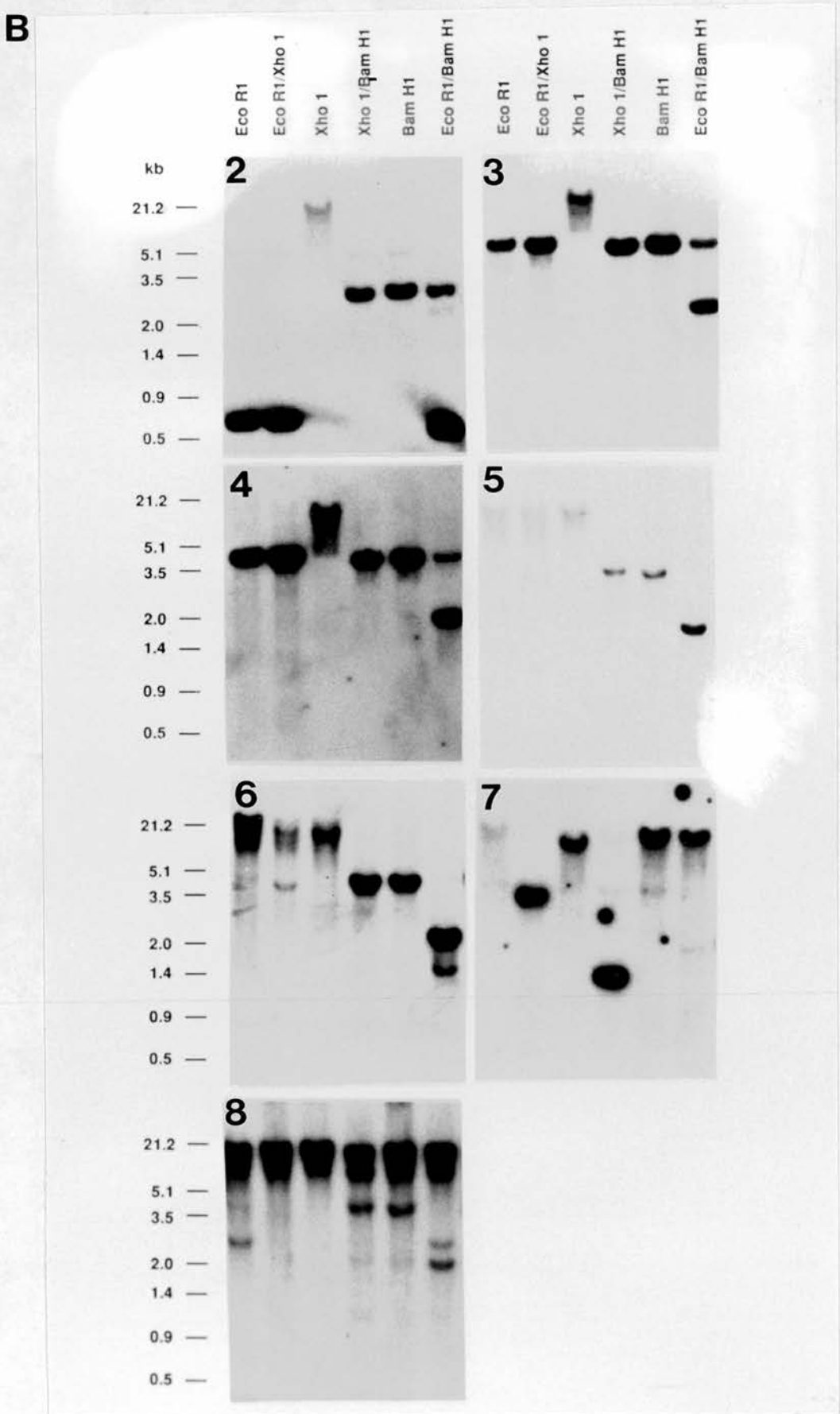


Fig. 7.3

Restriction mapping of the mouse dopamine D2-R gene. Fig. 7.3A: Organisation and restriction map of the mouse dopamine D2-R gene showing the regions spanned by the isolated genomic clones determined by restriction digestion with Xho1 (X), EcoR1 (E) and BamH1 (B). The exons were ordered on the restriction map using Southern blot analysis as depicted in Fig. 7.3B on the following page. Exons are represented by boxes, the alternatively spliced exon (Exon 6) is shown in red. Fig. 7.3B: Autoradiograph showing Southern blot hybridisation of genomic clone 8 to ^{32}P end-labelled oligonucleotide probes spanning the intronic sequence of the dopamine D2-R gene. The nucleotides represented by each probe were as follows: (i) Exon 2 [165 to 182]; (ii) Exon 3 [292 to 309]; (iii) Exon 4 [502 to 519]; (iv) Exon 5 [615 to 632]; (v) Exon 6 [744 to 765]; (vi) Exon 7 [993 to 1010; (vii) Exon 8 [1157 to 1174].


B
Eco R1
Eco R1/Xho 1
Xho 1
Xho 1/Bam H1
Bam H1
Eco R1/Bam H1
kb
21.2
5.1
3.5
2.0
1.4
0.9
0.5
2
3
4
5
6
7
8

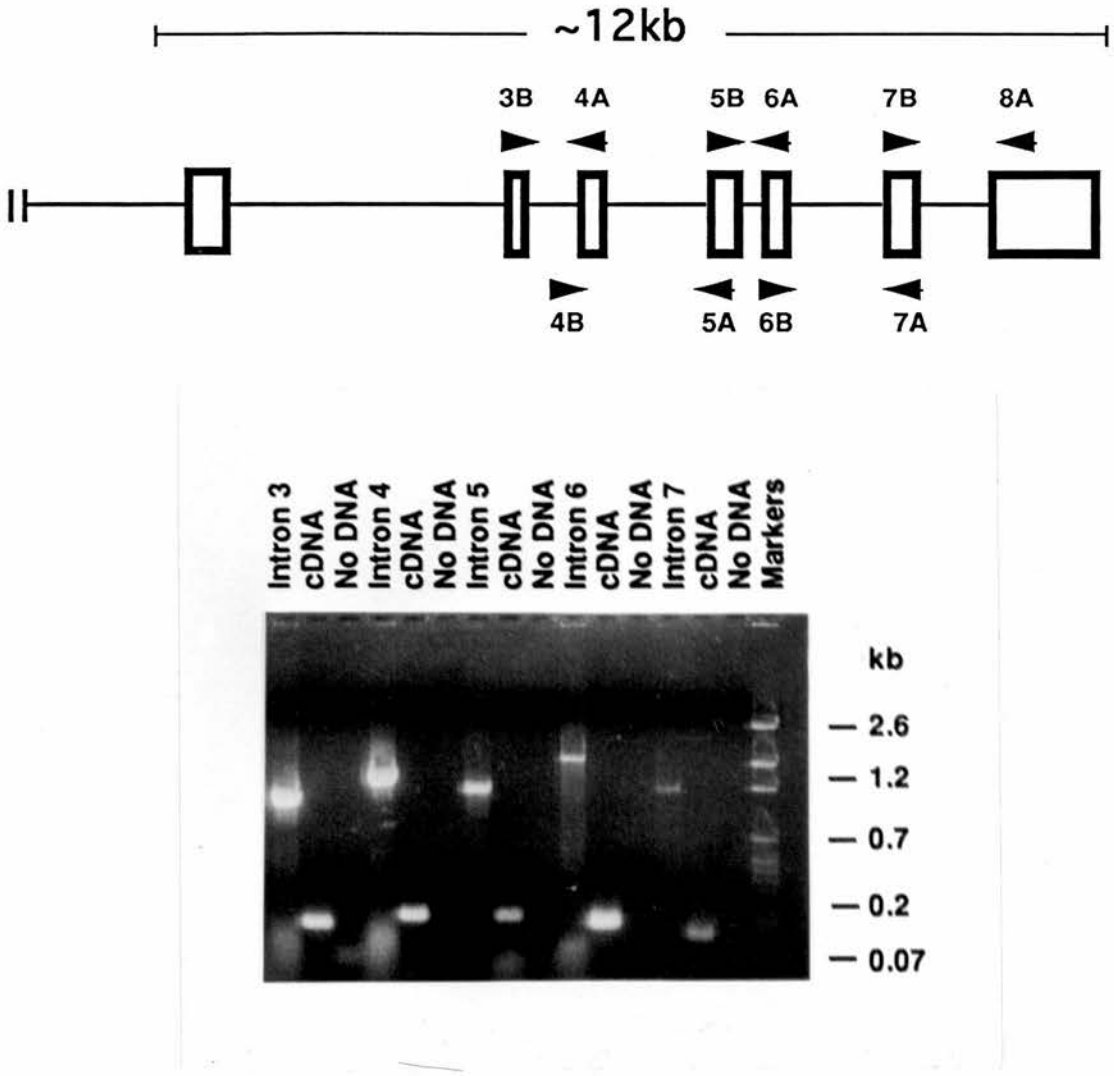


Fig. 7.4

Polymerase chain reaction (PCR) analysis of the mouse dopamine D2-R gene indicating the presence of introns: Fig. 7.4A: Diagram indicting the positions of the PCR primers (indicated by arrows) used to amplify the D2-R genomic clones. Introns are represented by lines and exons by boxes. Fig. 7.4B: Ethidium bromide-stained agarose gel showing PCR products amplified by the primer pairs in Fig. 7.4A. The pairs of PCR primers used were as follows: Intron 3 [3B/4A]; Intron 4 [4B/5A]; Intron 5 [5B/6A]; Intron 6 [6B/7A]; Intron 7 [7B/8A], see text for details. cDNA = rat D2-R cDNA (Eidne et al., 1989) as positive control, no DNA = negative control. The numbers to the right indicate standard DNA size markers in kilobases.

Donor	Acceptor	Intron Size (kb)
	....cccggccag^{-30}AGCCGT	>10.0
CTGGAG^{284}gtaggtcttgg....	....tttatctccag^{285}GTGGTG	~4.0
CGACAG^{395}gtgaggacat....	....tgcctttgcag^{396}GTACAC	~0.9
ACACAG^{531}**gc**aagtctgg....	....tattcccctag^{532}ACCAGA	~1.0
CTCAAG^{722}gtctccaacct....	...cactccacag^{723}GGCAAC	~0.9
AGAATG^{809}gtaagtgttca....	....tctctgcttcag^{810}GATCCT	~1.7
TTCTTG^{1140}gtgagtaagc....	.....ctcttcccag^{1141}GTGTGT	~1.0

Table 7.1

Donor and acceptor splice junctions of the mouse dopamine D2 receptor gene. Intron sequences are represented by lower case letters and dotted lines. The variant donor splice junction is in bold type.

position 1333 in Exon 8. The remainder of Exon 8 represented 3' untranlsated region.

7.4 Discussion

Organisation of the mouse gene:
This chapter has reported the cloning of the mouse dopamine D2-R gene spanning approximately 12kb of the genome and including 7 exons interrupted by 6 introns. The first untranslated exon was not identified. Reports of both the rat (O'Malley et al., 1990) and human (Gandelman et al., 1991) genes described a large (greater than 25kb) intron between Exons 1 and 2, and neither group obtained any overlapping clones in this region, despite extensive walking along the gene. The mouse D2-R gene structure was published during this project (Mack et al., 1991) and was also found to have a large intron in this region. More recently, a paper reporting the structure and linkage relationships of the human D2-R gene (Eubanks et al., 1992) indicated that the D2-R gene extends over 270kb in the human, with the first intron encompassing approximately 250kb. The positions of all introns in the D2-R gene are conserved between the rat, mouse and human (Fig. 7.5) and this close structural relationship between the D2-R genes of the three species suggests that the rat and mouse may also encode similarly large first introns. Interestingly, the human D2-R also appeared to contain 'nested' genes, ie. genes transcribed from within the region of the genome spanned by the D2-R (Eubanks et al., 1992).

Like the rat gene (O'Malley et al., 1990), the mouse gene encodes a variant donor splice site at the end of Exon 4 (a gc dinucleotide instead of the consensus gt). Detailed analysis by O'Malley et al. (1990) confirmed that this gc dinucleotide was an active splice junction and not a cloning or sequencing artefact. Mack et al., (1991) also reported a gc donor site for the mouse D2-R. The human gene encodes the usual gt dinucleotide at this position (Gandelman et al., 1991).

The D2-like genes (D2, D3 and D4) all contain introns, while the D1-like genes (D1 and D5) do not (Civelli et al., 1993). The intronic dopamine receptor genes are closely related to one another and also appear to be related to other members of the GPR superfamily such as rhodopsin (Nathans & Hogness, 1984), the opsins (Nathans et al., 1986),

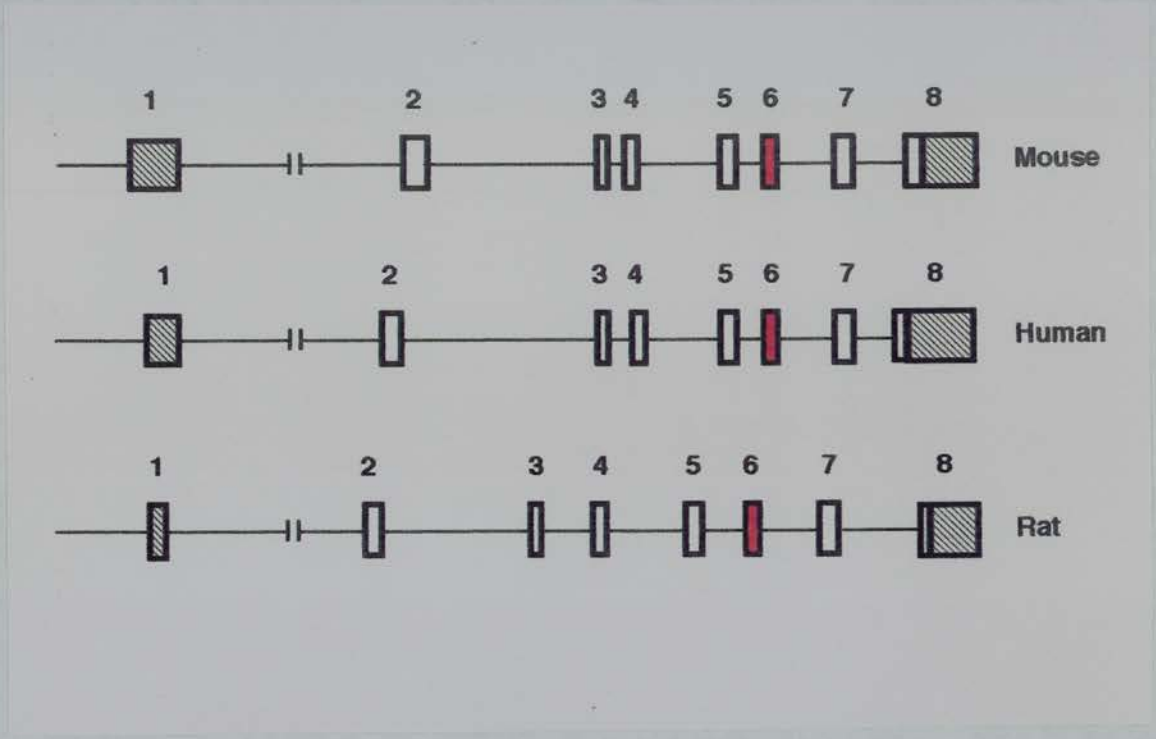


Fig. 7.5

Comparison of the organisation of mouse (Duthie, unpublished results; Mack et al., 1991), human (Gandelman et al., 1991) and rat (O'Malley et al., 1990) dopamine D2-R genes. Exons are represented by boxes, introns are represented by lines. Shading indicates untranslated sequence. The alternatively spliced Exon 6 is depicted in red.

the substance K receptor (Gerard et al., 1990) and the substance P receptor (Gerard et al., 1991), based on the conserved positions of several of their introns (Probst et al., 1992).

Alternative splicing of the mouse D2-R gene

Analysis of the mouse D2-R gene indicated that the extra 29 amino acids found inserted in the third cytoplasmic loop of the D2-R_{444} (Eidne et al., 1989; Giros et al., 1989; Rao et al., 1990; Chio et al., 1990; Grandy et al., 1989; Dal Toso et al., 1989; O'Malley et al., 1990) was encoded on a separate exon, Exon 6 (Fig. 7.4), as was found for the rat and human genes. Mack et al. (1991) used reverse-transcriptase PCR to demonstrate that Exon 6 could be transcribed or alternatively spliced out, to produce either a D2-R_{444} or D2-R_{415} in the mouse. They found that in the mouse, the long form of the receptor was predominant in brain, and could detect no expression of the short form of the receptor in mouse pituitary, a similar finding was reported in the rat (Eidne et al., 1989). Other groups, however, have found both isoforms in the pituitary in human (Dal Toso et al., 1989), rat (Giros et al., 1989; O'Malley et al., 1990) and mouse (Montmayeur et al., 1991) with the long form as the most abundant transcript.

The presence of an extra 29 amino acids in the third cytoplasmic loop does not appear to influence ligand recognition by the D2-Rs (Grandy et al., 1989; Giros et al., 1989), but is more likely to affect G-protein coupling, as this region has been implicated in second messenger activation by GPRs (see Chpt. 2.6.18). The D1-like and D2-like receptors each activate two types of signal transduction pathways. One is obligatory and is found in all cell types in which the receptor is expressed. The other depends on the cellular background of the receptor (Vallar et al., 1990; Civelli et al., 1993). The obligatory pathway for the D2-Rs involves inhibition of adenylate cyclase activity via the G-protein, $G\alpha_i$. In GH4C1 pituitary tumour cells, the D2-R inhibits adenylate cyclase, does not affect phosphoinositide hydrolysis, and induces a decrease in $[Ca^{2+}]_i$ due to a hyperpolarising effect mediated by the activation of K^+ channels. In Ltk$^-$ cells, the activated D2-R produces an increase in $[Ca^{2+}]_i$, partly due to the release of Ca^{2+} from intracellular Ca^{2+} stores following the stimulation of the PI pathway, and partly due to an influx of extracellular

Ca^{2+} (Civelli et al., 1993). In Chinese hamster ovary cells (CHO cells), D2-Rs mediate the release of arachidonic acid by a mechanism involving protein kinase C, independently of the inhibition of adenylate cyclase (Kanterman et al., 1991). This ability to activate different second messenger pathways implies that the D2-R either couples to one G-protein that can activate multiple second messenger pathways, or that the receptor can couple to more than one G-protein (Birnbaumer, 1990; Milligan, 1993), see Chpt .2, Fig. 2.10.

A recent study reported that the extra 29 amino acids in the long form of the D2-R allow the receptor to couple to a specific G-protein isoform, $G\alpha_{i2}$ (Montmayeur et al., 1993). This confirms that the two D2-R isoforms are functionally distinct, exerting their specific effects at the level of intracellular signalling.

The D2-R and disease

One of the major reasons for cloning and characterising the D2-R gene is related to the importance of the D2-R in several disease states. The D2-R has been implicated in the pathogenesis of some pituitary tumours such as prolactin-secreting adenomas (prolactinomas). Hyperprolactinaemia can lead to infertility as high levels of prolactin result in the partial suppression of GnRH release and loss of GnRH pulsatility. High levels of prolactin also interfere with the action of LH and FSH on the gonad, cause an increase in adrenal androgen secretion and result in the inhibition of positive oestrogen feedback on GnRH and LH secretion in women (Fig.7.6) (Thorner, 1987).

Most prolactinomas respond to dopamine agonists, such as bromocriptine, as shown by a decrease in prolactin release and by the inhibition of tumour growth (Bevan et al., 1992). Some tumours (approximately 11%) however, do not shrink in response to dopamine agonist therapy (Bevan et al., 1992) although many of these do show a reduction in prolactin secretion. A study by Pellegrini et al., (1989) found that 5 resistant prolactinomas had reduced numbers of D2-Rs, and also that dopamine unusually stimulated adenylate cyclase in these tumours. Disruptions at different stages in the dopaminergic regulation of prolactin secretion may be responsible for tumours that are resistant to dopamine therapy. Mutations in the D2-R itself may affect ligand-binding or cause

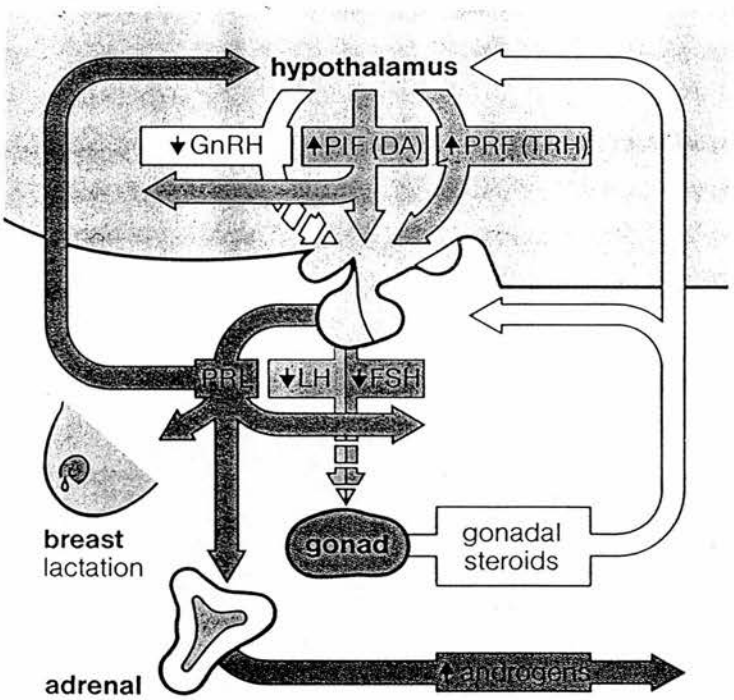


Fig. 7.6

Mechanisms of hyperprolactinaemic hypogonadism. Hyperprolactinaemia causes hypogonadism by several mechanisms: (i) High prolactin (PRL) levels lead to partial suppression of GnRH release, as well as loss of its pulsatility, and (ii) prolactin also interferes with the action of LH and FSH on the gonad. (iii) Prolactin causes an increase in adrenal androgen secretion, and (iv) leads to inhibition of positive oestrogen feedback on GnRH and LH secretion. PIF = prolactin inhibiting factor, PRF = prolactin releasing factor (from Thorner, 1987).

the receptor to activate G_s instead of G_i, resulting in the stimulation of adenylate cyclase instead of its inhibition. If an aberrant receptor is involved in the pathogenesis of such tumours, it is imperative to first understand the organisation of the normal gene.

The D2-R has also been implicated in neuronal disorders including Parkinson's disease, schizophrenia, manic depression, and susceptibility to drug abuse and alcoholism (Civelli et al., 1991; Civelli et al.,1993). The identification of restriction fragment length polymorphisms in the D2-R gene has been used to look for linkage relationships between the D2-R and these pathologies (Uhl et al., 1993; Iversen, 1993). There is no direct evidence that schizophrenia is associated with the D2-R, although some recent research has implicated the D4-R, a D2-like receptor (Seeman et al., 1993). Alcoholism does appear to be linked to the D2-R (Blum et al., 1993) although the evidence is still controversial. Direct sequencing of DNA from patients suffering from alcoholism and comparison with the sequence and organisation of the normal gene may reveal mutations in the D2-R. It would then be informative to look at similar mutations in mouse models of these human diseases.

7.5 Summary

This chapter has reported the cloning and organisation of the mouse dopamine D2-R gene. The coding region of the gene spans approximately 12kb of the mouse genome and consists of 7 exons (Exons 2 to 8) interrupted by six introns. The untranslated Exon 1 was not identified. It is reported to be separated from the rest of the gene by a very large intron (greater than 250kb in the human) thus it is technically impossible to isolate the entire gene in one clone. The positions of all introns are conserved between the mouse, rat and human D2-Rs. All three species are capable of alternative splicing of Exon 6 which encodes 29 extra amino acids in the third cytoplasmic loop, a region implicated in G-protein coupling. Knowledge of the sequence and organisation of the D2-R will be useful in comparing the genes from patients suffering from the various neuronal and endocrine pathologies associated with the D2-R. The mouse may prove a useful animal model for studying some of these diseases.

8 General Discussion

The molecular cloning of increasing numbers of G-protein-coupled receptors and their subtypes has had an enormous impact on the understanding of the systems in which they operate. GPRs are involved in transducing signals to every major system in the body, and are often targets for drugs used in the clinical therapy of a wide variety of human diseases. Much of the research in the GPR field has been stimulated by pharmaceutical companies interested in developing drugs that act as selective agonists or antagonists for specific receptors. This requires specialist knowledge of the structure/function relationships of the GPRs with their ligands and second messenger pathways.

The GPRs described in this thesis, the TRH-R and dopamine D2-R, occupy key positions in physiological, and potentially pathological, systems and there is an obvious need for the development of selective agonists and antagonists for clinical use. It is important to study the structure/function relationships of human, as well as animal receptors, as relatively minor differences in the binding site of GPRs can affect the response of the receptor to a particular drug. The replacement of one amino acid residue between the human and rat serotonin 5-HT_{1B} receptors produced major pharmacological variation in the responses of the two receptors (Oksenberg et al., 1992). Sequence information from human GPRs is also critical when looking for receptor mutations that cause human disease, such as the constitutively activating mutations in the TSH-R (Parma et al., 1993) and LH-R (Shenker et al., 1993) discussed in Chpt. 2.6.30.

The cloning and characterisation of the human TRH-R (Chpt. 4) has revealed significant primary sequence differences in the cytoplasmic COOH tail of the receptor as well as minor differences at the extracellular NH_2 terminus and in the third cytoplasmic loop with respect to the published rodent TRH-R sequences. Stable expression of the receptor in a transfected cell line is one of the first steps towards studying its structure/function relationships, and will allow comparisons to be made between the different TRH-R subtypes. Site-directed mutagenesis of the putative ligand binding domain in combination with radio-labelled binding studies will then provide further information as to exactly which residues

are involved in mediating the effects of agonists, antagonists and partial agonists. The action of some ligands as partial agonists is reported to be species-specific (Freidinger, 1989) further emphasising the importance of studying the human receptor.

Once the primary DNA sequence is known the 3-dimensional structure of GPR binding domains can be studied by molecular modelling using the reported structure of the seven transmembrane protein, bacteriorhodopsin (Henderson et al., 1990) as a reference protein. There are some limitations involved in using this approach: bacteriorhodopsin does not couple to G-proteins and there is no apparent sequence homology between this protein and GPRs. The mammalian opsins, however, are G-protein-coupled and are activated in the same way as bacteriorhodopsin (Ovchinnikov, 1982). Although major advances towards understanding the 3-dimensional interactions of receptors with their ligands has been achieved in this way, the models provide only a static picture of the receptor. A recent suggestion was that GPRs are not static molecules, but exist in a dynamic equilibrium. The 7 transmembrane helices are proposed to 'wobble' around hinge residues such as glycines or prolines found in key positions in the middle of some of the α-helices. The connecting extracellular and cytoplasmic loops act as structural buffers to maintain the equilibrium. In this model, ligand-binding might trigger a chain of reactions that disturbs the equilibrium, causing the receptor to adopt another stable conformation that allows G-protein coupling (Hibert et al., 1993).

Ligand binding analysis of the human TRH-R may help to develop non-peptide ligands that could be used in clinical practice. As a peptide, TRH has a short half-life and is rapidly degraded by metabolic peptidases. Non-peptide ligands with greater stability, oral availability and longer duration of action than endogenous TRH would therefore be useful. Most of the successes in this field have been achieved with peptides that bind to GPRs including the development of synthetic, non-peptide analogues for somatostatin and GnRH, the opioids and gastrin/cholecystokinin (Freidinger, 1989).

TRH and DA have functions other than their roles as hypothalamic releasing/inhibiting factors. Dopamine functions as a classical neurotransmitter in the central nervous system and may also

function in the heart and kidney (Civelli et al., 1993 for review). TRH appears to be widespread throughout the nervous system, gastro-intestinal system and reproductive tracts (Jackson, 1982; Sharif, 1985; Scanlon & Hall, 1989 for reviews). The TRH-R has been localised to rat (Zabavnik et al., 1993) and human brain (Eymin et al., 1993), rat retina (Satoh et al., 1993), testis (Feng et al., 1993) and human prostate (Chpt. 5) using Northern analysis and *in situ* hybridisation techniques. When considering analogue design for these receptors it is important to develop ligands that will activate only one particular pathway without affecting others. Obviously it is important to clone the receptors from these varied locations and to compare them with the known pituitary receptors as there may be significant differences that can be utilised. A human brain TRH-R does appear to be identical to the pituitary receptor (Matre et al., 1993), but it is equally possible that other receptor subtypes exist.

In the pituitary gland both DA and TRH appear to operate through more than one receptor subtype. There are at least two forms of the TRH-R in the rat pituitary generated by alternative splicing of a retained intron (de la Peña et al., 1992b), and one subtype each in mouse (Straub et al., 1990) and human (Duthie et al., 1993a, Martre et al., 1883; Yamada et al., 1993) with the possibility of further unidentified subtypes in these species. The D2-R expresses two receptor subtypes in the pituitary generated by alternative splicing of the D2-R gene (Eidne et al., 1989; Giros et al., 1989; Rao et al., 1990; Chio et al., 1990; Grandy et al., 1989; Dal Toso et al., 1989; O'Malley et al., 1990). DA and TRH are both capable of coupling to more than one second messenger pathway and there appears to be some form of cross-talk between the signalling systems activated by the two hormones, with respect to the regulation of prolactin secretion from pituitary lactotrophs (de la Escalera et al., 1992). The dissociation of DA from its receptor and the subsequent activation of the adenylate cyclase signalling pathway appears to potentiate the stimulatory effect of TRH on prolactin secretion (de la Escalera et al., 1992).

DA and TRH receptors are also expressed in pituitary thyrotrophs where they regulate the secretion of TSH. Prolactin and TSH secretion are not linked physiologically and so there must be specific factors in each cell type regulating the expression of these receptors and their signalling

pathways. It is also possible that there are different DA and TRH receptor subtypes operating in the two cell types to allow for the differential regulation of prolactin and TSH. Experiments have indicated that TRH stimulation of prolactin and TSH secretion appears to operate via different calcium-mediated mechanisms in lactotrophs and thyrotrophs respectively (Geras et al., 1982).

The cloning strategies employed to isolate these pituitary receptors have made it difficult to determine conclusively whether lactotrophs and thyrotrophs express different receptor subtypes. For example, the mouse TRH-R_{393} (Straub et al., 1990) was cloned from a thyrotroph cell line that produces TSH (TtT cells), while the two forms of the rat receptor, TRH-R_{412} and TRH-R_{387} (de la Peña et al., 1992b) were isolated from a clonal tumour cell line that secretes prolactin and growth hormone (GH3 cells). A third form of the rat receptor, TRH-R_{411}, was isolated from a pituitary cDNA library (Sellar et al., 1993) as was the human TRH-R_{398} (Duthie et al., 1993a; Yamada et al., 1993). These cDNA libraries, prepared from pituitary tissue, should represent transcripts expressed in more than one pituitary cell type, making it impossible to distinguish between lactotrophs and thyrotrophs. A human TRH-R was also isolated from a human brain cDNA library (Matre et al., 1993) but the brain is a large tissue with so many different cell types that it is impossible to represent all expressed transcripts in a single library. Northern blot analysis cannot be used to solve the problem either, and can only confirm that more than one receptor subtype is expressed in the pituitary as was found for both the DA-R and TRH-R.

Most functional studies with the TRH-R have been performed in GH3 cells in which TRH stimulates the secretion of prolactin by activating the phosphoinositide intracellular signalling pathway via the G-proteins, G_q/G_{11} (Hsieh & Martin, 1992). TRH has also been shown to activate the adenylate cyclase second messenger system via the stimulatory G-protein, G_s (Paulssen et al., 1992). It is becoming apparent that the genetic background of the host cell into which a receptor is transfected can greatly influence the signalling systems activated (Buckley et al., 1990; Milligan, 1993). In the case of the D2-R, the same transfected receptor can couple to the PI pathway or the adenylate cyclase pathway depending on the background of the host cell (Vallar et al., 1990), see discussion in Chpt. 7.

There are not only multiple subtypes for different receptors, but multiple subtypes of each of the components of the different signalling pathways within the cell. The regulation of gene expression is one of the central means of controlling metabolic pathways in a temporal and tissue-specific manner and each cell-type is likely to have a specific pattern of expression of receptor subtypes, G-proteins and effector enzymes.

Another problem observed with the use of clonal cell lines in receptor analysis is the commonly observed over-expression of a particular receptor with respect to the *in vivo* situation (Buckley et al., 1990). High levels of receptor expression may distort the signalling responses in the cell making it difficult to draw relevant conclusions from functional data. In the case of TRH, the activation of large numbers of receptors could result in the cell being flooded with calcium, overloading the system, and disturbing the delicate balance between different components of the signalling pathway (L Anderson: personal communication).

Not only are the components within a cell important for receptor function and regulation, but *in vivo*, external factors are also involved in receptor regulation. For example, peripheral hormones such as oestrogens and glucocorticoids affect the responses of lactotrophs to TRH and DA, and thyroid hormones feedback on thyrotrophs inhibiting the secretion of TSH (Burrow, 1991). A study on the effects of thyroid hormones *in vivo* and in GH3 cells gave different results. *In vivo,* thyroid hormones appeared to down-regulate TRH-Rs at the level of transcription, while *in vitro*, down-regulation at this level was not observed and may occur post-translationally (Yamada et al., 1993). These findings demonstrate the importance of considering whether information obtained from receptor structure/function analysis in transfected cells provides accurate information with respect to the physiological situation.

Factors affecting the functional regulation of receptors may also dictate which receptor subtype is expressed in a particular cell, or there may be other components of the cell which are themselves temporally and tissue-specifically regulated. It is likely that a complex combination of factors is responsible for determining the response of a cell to a particular ligand. The D2-R subtypes are transcribed from a single gene as a result of an alternative splicing event in the third cytoplasmic loop (Giros et al., 1989; Rao et al., 1990; Chio et al., 1990; Grandy et al., 1989; Dal Toso et

al., 1989; O'Malley et al., 1990). These two subtypes have recently been shown to be functionally diverse, the long form of the receptor having an extra cassette of 29 residues that enables it to couple to a specific G-protein isoform (Montmeyeur et al., 1993). The TRH-Rs all exhibit a high degree of sequence homology except at the 3' end of the COOH tail where all receptor subtypes show sequence divergence after a specific residue. This finding suggests that a similar alternative splicing event may occur in the TRH-R. Structural analysis of the TRH-R gene lent some support to this theory, at least in the mouse (Chpt. 6) although no second form of the mouse TRH-R has yet been reported. PCR analysis of the rat gene (de la Peña et al., 1992b) and cloning of the human gene (Yamada et al., 1993) has revealed different genomic organisation of the coding regions for the TRH-R gene of all three species. The mouse appears to have one intron in the coding region at the extreme 3' end of the COOH tail (Duthie et al., 1993b). The rat gene seems to have no extra intronic sequence, encoding a 'retained intron' within the coding sequence instead, with the 3' junction of this 'intron' occurring in the same position as the mouse COOH tail intron (de la Peña et al., 1992b). The human TRH-R gene has an intron in the third cytoplasmic loop like the D2-R gene, but no reported intronic sequence at the COOH tail (Yamada et al., 1993).

This genomic organisation contrasts with the situation for the D2-R gene where the positions of the intron/exon boundaries have been strictly retained between the three species (O'Malley et al., 1990; Mack et al., 1991; Gandelman et al., 1991; Chpt. 7). The mouse TRH-R gene was analysed in part using PCR amplification, while information for the rat gene was entirely due to PCR analysis, and although results appeared clear-cut it is possible that small introns may have been overlooked or very large introns missed due to 'looping out' of intervening sequence. Another possibility is that there is more than one TRH-R gene. Since TRH, like DA, exerts more than one function in more than one location it is quite likely that there are several TRH-R genes expressed in different cell types as occurs in the different dopaminergic systems.

GH3 cells have been reported to coexpress the long and short forms of the rat TRH-R. Both receptors appear to have similar ligand binding characteristics in an oocyte expression system, but the variation observed at the COOH tail may be important in directing the coupling of

the receptor to a particular G-protein subtype as was reported for the D2-R (Montmeyeur et al., 1993) and the prostaglandin EP3 receptor (Namba et al., 1993). Expression of the two forms of the receptor in transfected cells may help to answer this question, although, as stated earlier, it is difficult to draw firm conclusions from this type of analysis.

In spite of the problems encountered with clonal cell lines and their various *trans*-acting factors they still provide the most useful method of studying receptor structural and functional relationships and can also be helpful in studying the *cis*-regulatory elements responsible for controlling receptor gene transcription. A standard approach is to transfect constructs of putative receptor regulatory elements linked to reporter genes into an appropriate host cell and to monitor expression of the reporter gene. This system will be useful for studying the TRH-R gene promotor elements such as the dinucleotide repeat sequence observed in the 5' untranslated region (Chpt. 6).

Ultimately, the role of a particular genomic sequence in regulating gene activity can be more completely assessed by constructing a transgenic mouse model. It is also becoming possible to use the transgenic approach to study mutated receptors in the whole animal, providing an opportunity to analyse functional receptor domains in a physiological situation. Establishing colonies of transgenic mice however, is time-consuming and expensive, while transfected cell lines are easily manipulated, provide valuable information very quickly and can also be used for screening appropriate constructs before their expression in transgenic mice.

The report published by Hibert et al., (1993) was entitled "This is not a G protein-coupled receptor", on the basis that all the evidence gathered from primary sequence alignments of GPRs, from ligand-binding studies, from site-directed mutagenesis and from 3-dimensional molecular modelling represents only the merest suggestion of an actual GPR. However, the increasing amount of structural and functional information becoming available together with the rapid advances in technical methods used for receptor analysis, suggest that there will come a time when a realistic model for the activation of GPRs and transduction of the signal within the cell may be confidently put forward, together with the statement 'This <u>is</u> a G-protein-coupled receptor!'.

Appendix I

Buffers and Stock Solutions

Biodyne A - High salt denaturing solution
0.5M NaOH, 1.5M NaCl

Biodyne A - Neutralising solution
0.5M Tris/HCl pH7.4, 1.5M NaCl

Chloroform
24:1 (v/v) mixture of chloroform and isoamyl alcohol. Stored at room temperature.

50XDenhardt's
2% (w/v) Ficoll™, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) bovine serum albumin (BSA), Pentax Fraction V. Stored at -20°C.

15% Formamide hybridisation buffer (cDNA probes)
200mM phosphate buffer pH7.2, 1mM EDTA, 7% (w/v) SDS, 1% (w/v) BSA, 15% (v/v) deionised formamide, 65ml water.

20% Formamide hybridisation buffer (oligonucleotide probes)
5XDenhardt's, 5XSSC, 50mM sodium phosphate pH 6.8, 1mM sodium pyrophosphate, 20% (v/v) deionised formamide. Add 100μg/ml freshly boiled, sonicated salmon sperm DNA just before use (Ullrich et al., 1984).

6XGel-Loading buffer (Agarose Gels)
0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in water, stored at 4°C.

LB (Luria-Bertani) Broth
12.5g Luria Broth Base in 500ml water (autoclaved).

LB soft top agarose

12.5g Luria Broth Base, in 500ml water (autoclaved), then add 4g SeaqKem agarose and re-autoclave.

LB bottom agar

18.5g Luria Agar in 500ml water (autoclaved).

Phosphate buffer pH7.2

Add 400ml of 1M Na_2HPO_4 solution to 150ml 1M $NaH_2PO_4.2H_20$ while stirring and monitoring pH (autoclaved).

ss-phenol (stock solution phenol)

Phenol is removed from -20°C, warmed to room temp. and melted at 68°C. Add hydroxyquinoline to final conc. of 0.1% (w/v). To melted phenol, add an equal volume of buffer (0.5M Tris/HCl, pH8.0) at room temp., stir for 15mins. and leave to separate. Remove upper layer and add an equal volume of 0.1M Tris/HCl, pH8.0, repeat extractions until pH of the phenol is >7.8. After equilibration, add 0.1% (v/v) 0.1M Tris/HCl, pH8.0 containing 0.2% β-mercaptoethanol. Store in dark at 4°C for 1 month.

Phenol/chloroform

1:1 (v/v) phenol & chloroform equilibrated with TE buffer (pH8.0). Stored at 4°C, protected from light.

20% SDS (sodium dodecyl suphate/lauryl sulphate)

20% SDS (w/v) in distilled water (autoclaved).

SM buffer

1.0M NaCl, 0.1M $MgSO_4.7H_2O$, 0.35M Tris/HCl pH 7.5, 0.01% (v/v) gelatin (autoclaved).

SOC medium

2% Bacto tryptone (w/v), 0.5% Bacto yeast extract (w/v), 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose.

20XSSC (sodium salt citrate)
3M NaCl, 0.3M Na_3 citrate.$2H_20$, 0.02M EDTA, pH 7.0 (autoclaved)

TM buffer
50mM Tris, pH7.4, 10mM $MgSO_4$. (autoclaved).

TE buffer
10mM Tris/HCl, pH7.8, 0.1mM EDTA (autoclaved).

10XTBE Electrophoresis Buffer
108g Tris base, 55g boric acid, 40ml 0.5M EDTA in 1l water (autoclaved).

Plasmid lysis buffer
20mM Tris/HCl pH8.5, 2mM EDTA, 1% (v/v) Triton-X-100.

Tissue culture solutions

Assay Buffer - receptor binding assay
20mM Tris/HCL pH7.4, 2mM $MgCl_2$.

Buffer A - inositol phosphate production assay/calcium imaging
NaCl (127mM), KCl (5mM), $MgCl_2$ (2mM), NaH_2PO_4 (0.5mM), $NaHCO_3$ (5mM), $CaCl_2$ (1.8mM), HEPES (10mM), and BSA (0.1%) pH 7.2.

DMEM medium (COS-1 cells)
To a 500ml bottle of Dulbecco's modified Eagle's medium (DMEM) add 10ml stock L-glutamine (0.3mg/ml final conc.), 5ml stock penicillin/streptocmycin (P/S) solution (100IU penicillin final conc., 100µg streptomycin final conc.) and 10% (v/v) dialysed heat inactivated foetal calf serum (HIFCS).

Trypsin-EDTA solution
Trypsin-EDTA is available as a X10 liquid solution (GIBCO-BRL) containing 5.0g trypsin (0.5% w/v) and 2g EDTA in 100ml and is stored at -20°C. The working concentration of trypsin is 0.1% w/v and the stock solution is

working concentration of trypsin is 0.1% w/v and the stock solution is diluted 1/5 with phosphate buffered saline (PBS). Aliquots are stored at -20°C.

Buffers and solutions for RNA extraction/Northern blots

Dye solution - RNA gel electrophoresis
7.5% (w/v) Ficoll 400, 0.1% (v/v) bromophenol blue, 1mg/ml ethidium bromide

10X MOPS/EDTA running buffer - RNA gel electrophoresis
8.36g MOPS (200mM), 0.744g EDTA (10mM), 0.82g sodium acetate (50mM), dissolved in 150ml double-distilled water and adjusted to pH 7.0 with sterile 5M NaOH.

Phenol (water-saturated)
100g highly purified stock solution phenol (Gibco-BRL) stored at -20°C. Before use, melt in water bath at 65°C, fill bottle with double-distilled water, shake well and leave overnight at 4°C to separate. Remove most of the water and store at 4°C for one month.

Sample buffer - RNA gel electrophoresis
200µl (10X) MOPS/EDTA running buffer, 1ml deionised formamide, 356µl formaldehyde (37% v/v)

Sodium acetate (2M)
Molecular biology grade sodium acetate (Sigma), adjusted to pH 4.0 with glacial acetic acid (autoclaved).

Solution D - RNA extraction
Dissolve 250g of Guanidinium thiocyanate (Fluka) in 293ml double-distilled water and add 17.6ml 0.75M sodium citrate pH 7.0 (previously prepared in baked glassware and autoclaved) and 26.4ml 10% (w/v) sarcosyl (at 65°C to dissolve). This solution can be stored at room temperature for three months. Before use, add 0.36ml β-mercaptoethanol

(in fume-hood) to 50ml of this stock solution in a sterile Falcon tube. This is now solution D and can be stored at room temperature for one month. Final solution: 4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% sarcosyl, 0.1M β-mercaptoethanol.

Buffers and solutions for *in situ* hybridisation

All glassware used must be washed and baked to inhibit RNAses and gloves must be worn to prevent contamination of RNAses from hands.

1M dithiothreitol (DTT)
1g DTT dissolved in 6.49g distilled water.

Hybridisation buffer
50% (v/v) deionised formamide, 10% (v/v) dextran sulphate, 4XSTE buffer, 1X Denhardt's solution, 125µg/ml yeast tRNA, 125µg/ml salmon sperm DNA, 10mM dithiothreitol (DTT) and distilled water. Filter through 0.2µM millipore filter and store at -20°C.

4% paraformaldehyde (make fresh)
400ml phosphate buffered saline (without Ca^{2+} or Mg^{2+}) heated to 65°C, add 16g paraformaldehyde (wear carbon-filter mask), with 1 to 2ml 5M NaOH to dissolve. When cool, pH with conc. HCl and filter before use through Nalgene filters under vacuum.

0.1M phosphate buffer (make fresh)
9g NaCl, 3.5g Na_2HPO_4, 11.88g $NaH_2PO_4.2H_20$, 0.1g thiomersalate in 1l sterile water, pH to 7.4 with 5M NaOH and filter before use.

Prehybridisation buffer
50% (v/v) deionised formamide, 4XSTE buffer, 1X Denhardt's solution, 125µg/ml yeast tRNA, 125µg/ml salmon sperm DNA, 10mM dithiothreitol (DTT) and distilled water. Filter through 0.2µM millipore filter and store at -20°C.

RNAse buffer
100mM Tris/HCL pH 8.0, 0.5M NaCl, 1mM EDTA pH 8.0 made up to 1l with distilled water (autoclaved).

20XSTE
0.1M NaCl, 10mM Tris/HCl pH8.0, 1mM EDTA pH8.0.

Triethanolamine (TEA) buffer
10.4g TEA, 700ml distilled water, pH 8.0 (autoclaved).

Buffers and solutions for F.I.S.H.

Hybridisation buffer
50% (v/v) deionised formamide, 10% (v/v) dextran sulphate, 1XSSC. Aliqoted and stored at -20°C.

Wash A
100ml 20XSSC, 250µl Tween 20 (BDH), 25g dried skimmed milk (Marvel) made to 500ml with dH_20. Mix on low heat until dissolved. Spin 50ml aliquots at 2000rpm for 10 mins. and use supernatant.

FITC-avidin solution.
FITC-avidin (Vector Labs), 5µg/ml. in Wash A.

Biotinylated anti-avidin solution
Biotinylated anti-avidin (Vector Labs), 5µg/ml in Wash A.

Appendix II

Chemical and Equipment Suppliers

Amersham International plc, Amersham Place, Little Chalfont, Bucks. HP7 9NA, U.K.

Appligene, Pinetree Centre, Durham Road, Birtley, Chester-le-Street, Co. Durham DH3 2T1, UK.

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Ave. Hemel Hempstead, Herts. HP2 7TD, UK.

Biosoft, Cambridge, Cambs, U.K.

Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Bell Lane, Lewes, E.Sussex BN7 1LG, UK.

Clontech - Cambridge BioScience, 25 Signet Court, Stourbridge Common Business Centre, Swann's Road, Cambridge CB5 8LA, UK.

Costar, 205 Broadway, Cambridge, MA 02139, USA.

Falcon, Becton Dickinson UK Ltd., Between Towns Road, Cowley, Oxford, UK.

Flow Laboratories Ltd., PO Box 17, Second Avenue, Industrial Estate, Irvine, Ayrshire, Scotland KA12 8NB.

Flowgen, FMC Bioproducts, High Wycombe, Bucks, UK.

GIBCO-BRL, Life Technologies Ltd., PO Box 35 Trident House, Renfrew Road, Paisley PA3 4EF, Scotland, UK.

Greiner Labortechnik, Greiner GmbH, Maybachstrasse 2, D-7443, Frichkenhausen, Germany.

Hybaid Ltd., 111-113 Waldegrave Road, Teddington, Middlesex, UK.

Invitrogen - British Bio-technology Products Ltd., 4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS, UK.

Joyce Loebl Ltd., Dukesway, Team Valley, Gateshead, Tyne and Weir, UK.

Kodak - IBI Ltd., 36 Clifton Road, Cambridge CB1 4ZR, UK.

Kontron Instruments, 52 Telford Road, Lenziemill, Cumbernauld, UK.

National Diagnostics, 305 Paton Drive, Atlanta, Georgia, USA.

NBL, Northumbria Biologicals Ltd., Enzymes Division, South Nelson Industrial Estate, Cramlington, Northumberland NE23 9HL, UK.

Novagen, 593 Science Dr. Madison WI 53711, USA

Nunc, A/S Nunc, Kamstrupvej 90, Kamstrup, DK-4000 Roskilde, Denmark.

O. Kindler GmBH & Co., Ziegelhofstraße 214, D07800 Freiburg, Germany.

Pall, BioSupport Division, Pall Process Filtration Ltd., Europa House, Havant Street, Portsmouth PO1 3PD, UK.

Peninsula, 611 Taylor Way, Belmont, CA 94002, USA.

Perkin Elmer Cetus, 761 Main Ave., Norwalk, CT 06859.

Promega Ltd., 2800 Woods Hollow Road, Madison, WI 53711-5399, USA.

Raymond A. Lamb, 6 Sunbeam Road, London, UK.

Promega Ltd., 2800 Woods Hollow Road, Madison, WI 53711-5399, USA.

Raymond A. Lamb, 6 Sunbeam Road, London, UK.

SEMAT, Semat Technical (UK) Ltd., 1 Executive Park, Hatfield Road, St. Albans, Herts. AL1 4TA, UK.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset BH17 7NH, UK.

Stratagene Ltd., Cambridge Innovation Centre, Cambridge Science Park, Milton Road, Cambridge CB4 4GF, UK.

Stratech Scientific, Luton, UK.

USB - United States Biochemical Corporation, Cambridge, UK.

Vilber Lourmat, 77202 Marne la Vallee, Cédex 2, France.

Whatman LabSales Ltd., Unit 1 Coldred Road, Parkwood, Maidstone, Kent UK.

Appendix III

Additional methods

Calcium image analysis

Intracellular calcium measurements

Before $[Ca^{2+}]_i$ measurements could be determined, cells first had to be loaded with the fluorescent calcium dye fura-2. Trypsinized single cells were plated on to sterile glass coverslips. After 2 days attached cells were washed (X2) with Buffer A (Appendix I). Cells were loaded with fura-2 AM ester (4μM final conc. in Buffer A) for 30min. at 37°C in a 5% CO_2 humidified incubator. Unincorporated dye was removed by washing (X3) with Buffer A. Coverslips were then transfered to the heated stage (37°C) of an inverted epifluorescence microscope (see below).

Stimulation of cells with TRH and $[Ca^{2+}]_i$ Imaging

Dynamic video imaging was carried out using the MagiCal hardware and Tardis software provided by Joyce Loebl Ltd. A Nikon Diaphot microscope with a X40 quartz oil immersion objective, operated in epifluorescence mode, was used to image the cells.

Fluorescent images were obtained by exposing cells to 340nm and 380nm light, alternated under computer control, at a speed of approximately 0.6Hz. The images, viewed at wavelengths of 510nm (with a 40nm wide band filter), were focused on to the face of an intensified charge-coupled device camera (Photonic Sciences, UK) interfaced in turn, to an analogue hardware averager and 8-bit analogue-to-digital converter, both operating at video frame rate. Typically, eight images were averaged at each wavelength. A similar number were collected for background images which were subsequently subtracted on a pixel-by-pixel basis from the image samples. These images were held in dynamic random access memory for subsequent processing and analysis.

Fluorescence excitation shifts occur when fura-2 binds calcium, i.e. the excitation efficiency increases at 340nm and 380nm. Ratios of values obtained at 340/380nm represent changes in $[Ca^{2+}]_i$. The 340/380nm ratio was calculated from averaged video frames on a pixel-by-pixel basis, and were proportional to the intracellular ionized Ca^{2+} concentration. Calibration was performed on cells loaded with dye which had been made permeable to extracellular Ca^{2+} using 1μM ionomycin. Exposure of cells to solutions containing either 10mM Ca^{2+} or 10mM EGTA provided an estimate of the minimal and maximal fluorescence at 340nm and 380nm. A dissociation constant of 225nm for fura-2 and Ca^{2+} at 37°C was used. Possible errors in $[Ca^{2+}]_i$ determination were estimated to be 5% from frame to frame, and were measured by collecting data from known standards of Ca^{2+}/EGTA buffers, containing 50-100μM fura-2 free acid. Within a single frame, the maximal pixel-to-pixel standard error (spatial variation) was less than 3% in concentration for an average of 4 frames, and this fell to under 2% for an average of 8 frames.

Data Analysis and Presentation

Software-based image analysis using MagiCal allowed quantitation of ionized Ca^{2+} either in the whole cell or in selected regions of the cell versus time. This was accomplished by the use of a light pen accessed into dynamic memory to define a pixel data-set on a given image frame and the software then automatically constructed a graphical presentation of Ca^{2+} concentration with time. AscII files of quantitative data could also be derived, and these were used to derive plots of $[Ca^{2+}]_i$ versus time for either single cells, or the averges of several cells.

Fluorescence *in situ* hybridisation (F.I.S.H.)

In situ hybridisation to chromosomes (F.I.S.H.) provides a direct approach to chromosome mapping. A probe is labelled with biotin by nick-translation. Following hybridisation, the probe is detected by incubation with avidin-FITC. Prior to hybridisation, approximately fifty normal male chromosome spreads are banded and photographed. Each photographed cell is re-located after hybridisation and examined by phase-contrast

microscopy. Any hybridisation signals present on the post-hybridisation chromosomes are marked in the appropriate position on the corresponding pre-hybridisation photographs.

Chromosome preparation

Using standard cytogenetic methods, metaphase chromosome spreads were obtained from two human males of normal karyotype. Chromosome spreads in marked slide areas were banded using the Lipsol method (1% Lipsol for 10-15 seconds, rinse with saline, 1:4 Leishmann's stain in pH6.8 buffer for 2.50 mins., rinse with pH6.8 buffer), mounted in buffer, blotted dry and photographed within a marked $2cm^2$ area. Coverslips were removed in buffer and the slides de-stained in an ascending series of alcohols and air-dried prior to hybridisation (Garson et al., 1987; Boyd et al., 1989).

DNA probe labelling

Probes were labelled with biotin-11-dUTP (Sigma) by nick-translation using the BRL Nick Translation Kit 8160SB, according to manufacturer's instructions, and precipitated with 4.6µl 3M sodium acetate pH5.2, 1µl 20mg/ml glycogen and 122µl cold ethanol. The reaction was vortexed to mix, centrifuged for 30min. at 12,000rpm and the supernatant removed. The pellet was freeze-dried and resuspended at a concentration of 100ng/µl in TE buffer.

In situ hybridisation

The method used was essentially that described by Pinkel et al. (1986) with modifications by Carter et al., (1992) although the pre-hybridisation step was omitted for these single copy probes. The probes were diluted to a concentration of 10 to 30ng/µl in hybridisation buffer (Appendix I). The probe (100ng/slide area) was applied to the slide, a coverslip added and sealed with Cow Gum. The probe and chromosomal DNA were denatured together by incubation at 80°C for 10min. and quenched in cold ethanol. Slides were incubated on a metal tray floating in a water-bath at 37°C overnight.

Post-hybridisation washing and detection

Coverslips were carefully removed from slides before soaking in 2X SSC for 5mins. The slides were washed for only 1.5mins in 50% formamide (in 2X SSC) at 42°C, and rinsed in 2X SSC for a further 5mins. before incubating in Wash A (Appendix I) at 37°C for 30mins. During the wash period, detection agents were diluted and incubated for 10mins. at room temperature, then microcentrifuged for 10mins. to pellet any precipitate and only the supernatants used.

To detect the biotinylated probe, 100µl FITC-avidin solution (Vector Laboratories) was pipetted on to the hybridisation area, which was overlaid with a coverslip and incubated at 37°C for 20mins. in a humidified box. Coverslips were removed and the slides washed for 3x5mins. in Wash A at 42°C. Following this wash, 100µl biotinylated anti-avidin solution (Vector Laboratories) was pipetted on to the hybridisation area, the slide coverslipped and incubated for 20mins. at room temperature in a covered (to prevent FITC fading) humidified box. The coverslips were again removed and the slides washed for 2x5mins. in Wash A at 42°C. Slides were again incubated with 100µl FITC-avidin solution at room temperature for 20mins. in a covered, humidified box. Washes were repeated for 2x5mins in Wash A at 42°C. The signal was amplified twice by two further incubations with biotinylated anti-avidin, followed by FITC avidin. Slides were rinsed 2x5mins. in 4X SSC containing 0.05% Tween-20 at room temperature ready for counter-staining.

Slides were briefly dehydrated in an ascending series of alcohols, air-dried and the chromosomes were counterstained by mounting the slides in 25µl antifade AF1 (Citfluor) containing 0.8µg/ml 4,6-diaminidino-2-phenylindole (DAPI) and 0.4µg/ml propidium iodide. The hybridisation area was overlaid with a coverslip and sealed with nail varnish.

Microscopy and analysis

For best results, the slides were stored at 4°C overnight before microscopy and were examined using a Zeiss Axioplan fluorescence microscope. FITC and propidium iodide were excited at 490nm (Zeiss filter combination 9) and the hybridisation signals appeared as yellow-green spots. Previously photographed, DAPI-stained cells were relocated using Zeiss filter

combination 1. Signals visualised on the post-hybridisation metaphases using filter set 9 were marked on the photographs of pre-hybridisation banded metaphases. Each signal was then plotted on a chromosome ideogram and the distribution of hybridisation in chromosome spreads was analysed statistically using the chi-square (χ^2) test.

Appendix IV

525 EAST 68th STREET, NEW YORK, N.Y. 10021

THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

MARVIN C. GERSHENGORN, M.D.
ABBY ROCKEFELLER MAUZE DISTINGUISHED PROFESSOR
OF ENDOCRINOLOGY IN MEDICINE
DIRECTOR, DIVISION OF MOLECULAR MEDICINE

(212) 746-6275
FAX (212) 746-6289

December 23, 1993

Sarah Duthie
MCR Reproductive Biology Unit
Centre for Reproductive Biology
37 Chalmers Street
Edinburgh EH3 9EW

Dear Sarah,

Congratulations again on a very nice research project.

I can offer the following observations regarding the mouse TRH-R gene. These data confirm your findings. Using a mouse genomic clone that we isolated from a different library than the one you used, we find the same size PCR products in our genomic and cDNA clones with the forward primer from extracellular loop 2 and reverse primers from transmembrane helix 7 or the carboxyl tail. Therefore, we also find no evidence for an intron in this region of the mouse gene.

Best of luck in your post-doc.

Sincerely yours,

Marvin C. Gershengorn

TOTAL P.01

Appendix V

Publications

Duthie, S.M., Taylor, P.L., Anderson, L., Cook, J. & Eidne, K.A. (1993a) Cloning and functional characterisation of the human TRH receptor. Molecular and Cellular Endocrinology **95**:R11-R15.

Duthie, S.M., Taylor, P.L. & Eidne, K.A. (1993b) Characterization of the mouse thyrotrophin-releasing hormone receptor gene: an exon corresponds to a deletion in the rat cDNA. Journal of Molecular Endocrinology **11**:141-149.

Morrison, N., Duthie, S.M., Boyd, E., Eidne, K.A., Connor, J.M. Assignment of the gene encoding the human thyrotrophin-releasing hormone receptor to 8q23 by fluorescence in situ hybridisation. Human Genetics (*in press*).

Note: Permission to include photocopies of the above papers has been obtained from the publishers and authors concerned.

K.A. Eidne
(Project supervisor)

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